

Identification and functional analysis of a phytoene desaturase gene from the extremely radioresistant bacterium *Deinococcus radiodurans*

Zhenjian Xu,[†] Bing Tian,[†] Zongtao Sun, Jun Lin and Yuejin Hua

Institute of Nuclear-Agricultural Sciences, Zhejiang University, 310029 Hangzhou, China

Correspondence
Yuejin Hua
yjhua@zju.edu.cn

The phytoene-related desaturases are the key enzymes in the carotenoid biosynthetic pathway. The gene encoding phytoene desaturase in the deinoxanthin synthesis pathway of *Deinococcus radiodurans* was identified and characterized. Two putative phytoene desaturase homologues (DR0861 and DR0810) were identified by analysis of conserved amino acid regions, and the former displayed the highest identity (68%) with phytoene desaturase of the cyanobacterium *Gloeobacter violaceus*. DR0861 gene knockout and dinucleotide-binding motif deletion resulted in the arrest of lycopene synthesis and the accumulation of phytoene. The colourless DR0861 knockout mutant became more sensitive to acute ionizing radiation and oxygen stress. Complementation of the mutant with a heterologous or homologous gene restored its pigment and resistance. The desaturase activity of DR0861 (*crtI*) was further confirmed by the assay of enzyme activity *in vitro* and heterologous expression in *Escherichia coli* containing *crtE* and *crtB* genes (responsible for phytoene synthesis) from *Erwinia uredovora*. In addition, the amount of lycopene synthesis in *E. coli* resulting from the expression of *crtI* from *D. radiodurans* was determined, and this had significant dose-dependent effects on the survival rate of *E. coli* exposed to hydrogen peroxide and ionizing radiation.

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INTRODUCTION

Deinococcus radiodurans is a red-pigmented, non-photosynthetic bacterium well known for its extraordinary tolerance to γ -rays, UV radiation, oxidizing agents and desiccation (Cox & Battista, 2005; Makarova *et al.*, 2001). The remarkable capacity of *D. radiodurans* to survive damage has been attributed to three mechanisms: prevention, tolerance and repair (White *et al.*, 1999), and especially to its efficient DNA repair mechanisms (Minton, 1994). Although current research is concentrated on the proteins related to DNA repair, and several important genes involved in DNA repair have been identified in *D. radiodurans* (Earl *et al.*, 2002; Hua *et al.*, 2003; Narumi *et al.*, 2004), the repair pathways employed by this organism remain unclear.

Although the mechanisms underlying the extraordinary resistance of this bacterium are still poorly understood, it was deduced that free radical scavengers are important contributors to the prevention mechanism, and have an important role in preventing DNA damage in *D. radiodurans* (Ghosal *et al.*, 2005; Tian *et al.*, 2004). *D. radiodurans*

contains superoxide dismutase (SOD), catalase (CAT) and organic hydroperoxide resistance protein (OHRP), which serve as free radical scavengers (Makarova *et al.*, 2001; Meunier-Jamin *et al.*, 2004). Recently, the role of an antioxidant metabolite pyrroloquinoline-quinone (PQQ) from *D. radiodurans* in *Escherichia coli* has been reported; PQQ conferred markedly increased protection against reactive oxygen species in *E. coli* (Khairnar *et al.*, 2003). Moreover, Daly *et al.* (2004) reported that non-enzymic Mn(II) ions might act as antioxidants and reinforce enzymic antioxidant systems that defend against oxidative stress during recovery.

Carotenoids are well known for their free radical scavenging activities. The protective role of carotenoids against oxidative damage is essential to various organisms including photosynthetic and non-photosynthetic species (Armstrong & Hearst, 1996). The radioresistance of deinococci and sarcinae appears to be associated with the pigmentation of the strains (Mosely, 1983). *D. radiodurans* R1 has been reported to synthesize a unique carotenoid product that was identified as deinoxanthin (Laurant *et al.*, 1997). However, few investigations concerning the antioxidant effects of this carotenoid have been done to date (Melin *et al.*, 1998), and its biosynthesis pathway remains unclear. Generally, carotenoid biosynthesis starts from the condensation of two molecules of geranylgeranyl diphosphate, which

[†]These authors contributed equally to this work.

Abbreviations: CAT, catalase; OHRP, organic hydroperoxide resistance protein; PQQ, pyrroloquinoline-quinone; ROS, reactive oxygen species; SOD, superoxide dismutase.

generates phytoene. This step is followed by four steps of desaturation catalysed by CrtI resulting in the synthesis of lycopene, or three-step desaturation catalysed by CrtP resulting in the production of neurosporene (Armstrong *et al.*, 1990; Ehrenshaft & Daub, 1994; Harada *et al.*, 2001). Further downstream reactions, including cyclization of lycopene, addition of keto groups and hydroxylation of cyclized carotene, lead to the formation of different products.

Results from comparative genomic analysis showed that the *D. radiodurans* genome includes several genes predicted to be involved in carotenoid synthesis (Makarova *et al.*, 2001). Two genes encoding ketolase (CrtO) and cyclase (CrtLm) in *D. radiodurans* were described recently (Tao & Cheng, 2004; Tao *et al.*, 2004). Further work needs to be done on other related genes to elucidate the deinoxanthin biosynthesis pathway in this bacterium. On the basis of sequence alignment and conserved amino acid analysis, we identified three genes as putative candidates for the phytoene desaturase of *D. radiodurans*. Among them, DR0810 showed 34% identity to the phytoene desaturase of *Rubrobacter xylanophilus*. Although DR0093 had 41% similarity to the carotenoid desaturase of *Nocardia farcinica*, a recent study has shown that DR0093 acts as a

β -carotene ketolase (Tao & Cheng, 2004). Another candidate, DR0861, exhibited the highest similarity to the phytoene desaturase sequence from the cyanobacterium *Gloeobacter violaceus*: about 68% global identity and nearly 80% similarity. Two highly conserved regions were detected in DR0861 and DR0810 genes: the putative dinucleotide-binding motif ($\beta\alpha\beta$ fold) in the N-terminal region (Fig. 1a) and the 'bacterial-type phytoene desaturase signature' at the C terminus (Fig. 1b).

In the present study, DR0861 and DR0810, the two putative phytoene desaturase genes in the *D. radiodurans* genome, were selected for investigation of their functions. Using gene inactivation and *in vivo* complementation, heterologous expression and *in vitro* enzyme activity assays, followed by HPLC analysis, we identified DR0861 as the gene encoding a typical bacterial-type phytoene desaturase (CrtI), whereas DR0810 was not involved in the deinoxanthin synthesis pathway. Our results also demonstrate that the carotenoid synthesis pathway contributes to the radioresistance and oxidative stress tolerance of *D. radiodurans*. In addition, lycopene synthesis resulting from the heterologous expression of the *crtI* gene had significant dose-dependent effects on the survival rate of *E. coli* exposed to hydrogen peroxide and ionizing radiation.

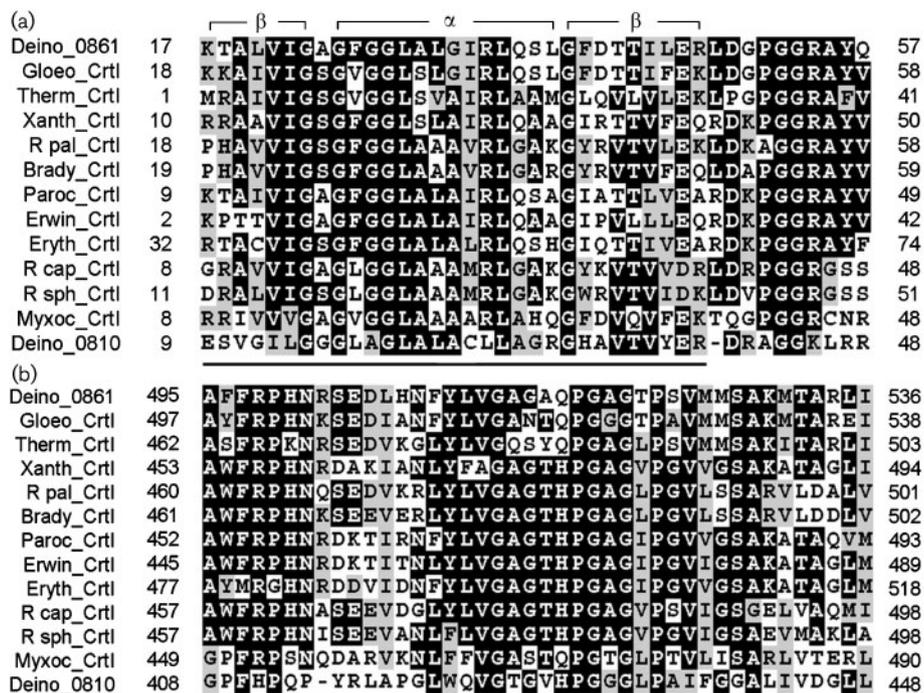


Fig. 1. Multiple sequence alignment of (a) the N-terminal region and (b) the 'bacterial-type phytoene desaturase signature' C-terminal region from *D. radiodurans* R1 (Deino), *Gloeobacter violaceus* PCC 7421 (Gloeo), *Thermus thermophilus* HB8 (Therm), *Xanthobacter* sp. Py2 (Xanth), *Rhodopseudomonas palustris* CGA009 (R pal), *Bradyrhizobium* sp. ORS278 (Brady), *Paracoccus* sp. MBIC1143 (Paroc), *Erwinia uredovora* (Erwin), *Erythrobacter longus* (Eryth), *Rhodobacter capsulatus* (R cap), *Rhodobacter sphaeroides* (R sph), and *Myxococcus xanthus* (Myxoc). The highly conserved dinucleotide binding motif ($\beta\alpha\beta$ fold) and the consensus signature sequence are underlined. Identical and similar amino acids are indicated by black and grey boxes, respectively.

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
Δ crtI	<i>D. radiodurans</i> DR0861 gene knockout mutant	This work
Δ crtI _{DO}	<i>D. radiodurans</i> DR0861 gene (amino acids 19–69) deletion mutant	This work
RI _O	R1 with pRADI _{DR}	This work
KI _{DR}	<i>D. radiodurans</i> Δ crtI complement with pRADI _{DR}	This work
KI _{EU}	<i>D. radiodurans</i> Δ crtI complement with pRADI _{EU}	This work
<i>E. coli</i>		
DH5 α	Host for cloning vectors	Laboratory stock
BL21(DE3)	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS</i> ($r_B^- m_B^-$) <i>gal</i> λ (DE3)	Novagen
BL _I	BL21 containing expression plasmid pET28I	This work
BL _{EB}	BL21 containing plasmid pACCRT-EB	This work
BL _{EIB}	BL21 containing plasmid pACCRT-EB and pET28I	This work
Plasmids		
pGEM-T Easy	Cloning vector (Ap ^r)	Promega
pET28a	Expression vector (Km ^r)	Novagen
pTK	pGEM-T Easy vector containing <i>crtI</i> gene flanking sequence and kanamycin cassette (Ap ^r)	This work
pRADZ3	<i>E. coli</i> - <i>D. radiodurans</i> shuttle vector carrying R1 <i>groEL</i> promoter (Ap ^r Cm ^r)	Meima & Lidstrom (2000)
pRADK	pRADZ3 derivative in which <i>lacZ</i> is replaced by the kanamycin gene (Ap ^r Km ^r Cm ^r)	Laboratory stock
pRADI _{DR}	pRADZ3 derivative in which <i>lacZ</i> is replaced by the <i>crtI</i> gene of <i>D. radiodurans</i> (Ap ^r , Cm ^r)	This work
pRADI _{EU}	pRADZ3 derivative in which <i>lacZ</i> is replaced by the <i>crtI</i> gene of <i>Er. uredovora</i> (Ap ^r , Cm ^r)	This work
pET28I	pET28a expression plasmid containing <i>NdeI</i> - <i>XhoI</i> fragment of <i>crtI</i> (Km ^r)	This work
pACCRT-EB	pACYC184 containing <i>crtE</i> and <i>crtB</i> genes of <i>Er. uredovora</i> (Cm ^r)	Misawa <i>et al.</i> (1990)
pACCRT-EIB	pACYC184 containing <i>crtE</i> , <i>crtB</i> and <i>crtI</i> genes of <i>Er. uredovora</i> (Cm ^r)	Misawa <i>et al.</i> (1995)

METHODS

Strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. *D. radiodurans* R1 (ATCC 13939) was used as the wild-type strain and for construction of mutants and was grown in TGY medium as described previously (Mosely, 1983). Kanamycin (20 μ g ml⁻¹) or chloramphenicol (3 μ g ml⁻¹) was added to the medium if required. *E. coli* strains were cultured in LB broth.

Sequence analysis. Comparisons of sequences with various databases were carried out with FASTA and the default parameters of BLASTP (<http://www.ncbi.nlm.nih.gov>). Multiple alignments were performed with the CLUSTALW program at the EMBL (<http://www.ebi.ac.uk/CLUSTALW>). Subsequent adjustments of these alignments were done manually.

Construction of *D. radiodurans* mutants. Mutants were constructed by double crossover recombination of a kanamycin resistance cassette into the genome. The sequence of *D. radiodurans* R1, obtained through NCBI (<http://www.ncbi.nlm.nih.gov/Database>), was used to design the primers (Table 2). A 900 bp *Bam*HI fragment upstream and a 900 bp *Hind*III fragment downstream of the targeted genes were amplified using PCR. The kanamycin resistance cassette containing the *groES* promoter was obtained from pRADK, a shuttle plasmid modified from pRADZ3 (Gao *et al.*, 2005; Meima & Lidstrom, 2000). The upstream and downstream fragments were digested with *Bam*HI and *Hind*III respectively, and ligated to the *Bam*HI-*Hind*III fragment of the kanamycin resistance cassette. The joint product of the three fragments was further amplified by PCR and ligated into the pGEM-T Easy vector to yield pTK (Table 1). The plasmid was linearized with *Eco*RV and transformed into *D. radiodurans* R1 with CaCl₂ as described previously (Kitayama *et al.*, 1983). Mutants were selected on TGY plates containing an appropriate antibiotic. The N-terminal motif (amino acids 19–69)

deletion mutant was constructed by the same method and designated Δ crtI_{DO}. All the recombinants were verified by PCR and sequencing.

Plasmid construction. We constructed two complementation plasmids containing intact phytoene desaturase gene homologues from *D. radiodurans* and *Erwinia uredovora*, respectively. A 1647 bp region containing the intact DR0861 gene was amplified by PCR, digested with *Spe*I and *Bam*HI, then ligated into pRADZ3 to create pRADI_{DR} (Table 1). Another 1489 bp fragment carrying the complete *Er. uredovora* *crtI* gene was obtained from the plasmid pACCRT-EIB (Misawa *et al.*, 1995). The fragment was digested with *Eco*RV and *Bam*HI and ligated into pRADZ3, and the resulting plasmid was named pRADI_{EU} (Table 1).

A plasmid for overexpression of DR0861 in *E. coli* was also constructed. The intact phytoene desaturase gene homologue was amplified by PCR with engineered *Nde*I and *Xho*I restriction sites, then ligated into pET28a to create the expression plasmid pET28I. The fragment was sequenced to confirm the correct sequence and orientation. Plasmid pET28I was transformed into *E. coli* BL21(DE3) and the resulting strain was named BL_I. The *E. coli* transformant carrying plasmid pACCRT-EB (Misawa *et al.*, 1990) was named BL_{EB}. The strain transformed with pET28I and pACCRT-EB was named BL_{EIB}.

Analysis of carotenoids. Briefly, carotenoids from *D. radiodurans* were extracted as follows: exponential-phase cell pellets obtained from 50 ml cell culture were extracted three times with 1 ml acetone/methanol (7:2, v/v) in the dark and the supernatant was collected. For the extraction of carotenoids generated in *E. coli*, cells carrying pACCRT-EB and/or pET28I were incubated in 100 ml LB broth with shaking at 37 °C. After the addition of 0.5 mM IPTG and subsequent incubation for 6 h, cells were harvested by centrifugation at 5100 g for 15 min. Carotenoids in *E. coli* were extracted by the method described above.

Table 2. Sequences of the primers used in this study and their relative positions in the *D. radiodurans* R1 strain genome

Nucleotides in lower case show changes that were made in the sequence to engineer restriction sites used for cloning. Restriction sites are in bold. Base numbering in the *D. radiodurans* R1 genome is as in the NCBI database. up, upstream primer; down, downstream primer.

Primer	Sequence (5'–3')	Position
5' DR0861 _{up}	cggtgtgcttgcGACCTCAAAAACCCCAT	1851–1868
3' DR0861 _{up}	ctgcat GGATCC GTCATACGGATTCCGCTT	2993–2810
5' DR0861 _{down}	atgctg AAGCTT GACAGTAAACCTCGGAAG	4458–4475
3' DR0861 _{down}	ccatattgctaGGGTCGCAACTGTTTTTCG	5353–5370
5' 0861Domain _{up}	gttgctgtggtcTTGCCGGGTAGATT	3562–3575
3' 0861Domain _{up}	taataa GGATCCCCG ACCGTGTGACG	4236–4250
5' 0861Domain _{down}	tgctta AAGCTT GCGGGCATAGGGCGAAG	4410–4426
3' 0861Domain _{down}	ttaggctcattgACGAGGCGGGAAAC	5275–5288
5' DR0810 _{up}	gcagcatggtgtCTGACCGGGCGGTTGTAT	7407–7424
3' DR0810 _{up}	ttatta GGATCC TGGCCGCGCGCCAGGAGG	8167–8184
5' DR0810 _{down}	gtatgt AAGCTT TCGATTCCCGAACATCC	9961–9978
3' DR0810 _{down}	tagtaatgccttCTGCTCAACCTGCTCACCTC	10601–10620
5' Complement _{DR}	ggcgtg ACTAGT ATGACATCTGCACTTCCT	2811–2828
3' Complement _{DR}	ttgatt CATATG TCAGCGCCGGATGTCC	4442–4457
5' Complement _{EU}	gggtga ACTAGT ATGAAACCAACTACGG	
3' Complement _{EU}	ggtgtg GATATC TATCATATCAGATCCTCC	
5' Expression	gtggtc CATATG ATGACATCTGCACTTCCT	2811–2828
3' Expression	tattat CTCGAG TCAGCGCCGGATGTCC	4442–4457
5' Kanamycin	CACACAGGAAACAGCTATGACCATGATTA	
3' Kanamycin	acagac GGATCC TAGAAAACTCATCGAGCATC	

Carotenoids in the pooled extracts were analysed by HPLC using a Waters Alliance 2690 system and a Hypersil C₁₈ column (250 × 4.6 mm, 5 µm, Alltech). A mixture of acetonitrile/methanol/2-propanol (40:50:10, by vol.) was used as the mobile phase. The eluted fractions were monitored using a Waters 996 photodiode array detector scanning from 200 to 800 nm. The flow rate was 0.8 ml min⁻¹. Carotenoids were identified by retention time, features of absorption spectra and by comparison with standard compounds or with reported data. The amount of lycopene generated in *E. coli* transformants was determined from the area under the peak detected at 470 nm using a calibration curve obtained with a lycopene standard (Sigma).

In vitro reaction and enzyme activity assay. The substrate carotene (phytoene) was extracted from *E. coli* containing pACCRT-EB by the method described above. The hydrophobic carotene was treated with soybean L- α -phosphatidylcholine (20%, w/v) to make a suspension and processed as previously described (Schneider *et al.*, 1997). The recombinant enzyme was produced in *E. coli* BL21(DE3) with pET28I and purified by a method described previously (Fraser *et al.*, 1992). Enzyme activity was assayed as follows. The reaction mixture (0.5 ml) contained the appropriate substrate (approx. 4 µg), 1.5 µM NADP and 30 µg purified enzyme in 100 mM Tris/HCl buffer (pH 7.9). The reaction was established by adding catalase (20 000 U ml⁻¹), glucose (2 mM) and glucose oxidase (20 U ml⁻¹). Incubation was in the dark at 37 °C for 4 h with shaking at 200 r.p.m. The reaction was terminated by adding methanol (1 ml) containing 6% (w/v) KOH and heating at 60 °C for 15 min. The products formed were extracted from the incubation mixture with diethyl ether/light petroleum (1:9, v/v). The above solvent phase was evaporated to dryness and the residue was redissolved in cool acetone/methanol (7:2, v/v). The reaction products were identified by HPLC using a Waters 600 system and a Hypersil ODS2 column (250 × 4.6 mm, 5 µm, Elite). The other conditions were as described in the preceding section.

Measurement of cell survival rate. The survival of *D. radiodurans* exposed to H₂O₂ and γ -radiation was determined by methods described previously (Carbonneau *et al.*, 1989; Funayama *et al.*, 1999). For the survival rate of *E. coli* exposed to γ -rays, cells grown to an OD₆₀₀ of about 0.5 were induced with 0.5 mM IPTG for an additional 6 h, and then were subjected to irradiation. The survival rate of *E. coli* treated with hydrogen peroxide was assayed as described previously (Konola *et al.*, 2000) with some modifications. Cultures of *E. coli* were induced with 0.5 mM IPTG for 6 h, then 30% H₂O₂ solution was added to the cultures to obtain final concentrations of 1, 2, 3 and 4 mM. No H₂O₂ was added to the control culture. After incubation with shaking at 200 r.p.m. for 15 min, catalase (Sigma) was added in excess (100 µg ml⁻¹) to each culture to inactivate H₂O₂. The cells were diluted appropriately, spread on TGY agar plates and incubated at 37 °C for 20 h. Survival rate was expressed as the number of colonies obtained from treated samples as a percentage of the number for untreated controls.

Statistical analysis. For cell survival experiments, experiments were performed four times, with three replicates in each experiment. The values were represented as mean \pm SD ($n=12$). Results were assessed by Student's *t*-test using Microsoft Excel, and $P<0.05$ was considered significant.

RESULTS

Phenotypes of DR0861 and DR0810 knockout mutants

The two putative phytoene desaturase genes were knocked out by using a kanamycin resistance cassette. The mutants

were confirmed by genome PCR, restriction digestion and DNA sequencing. In contrast to the red-pigmented wild-type strain, the DR0861 null mutant ($\Delta crtI$) turned out to be colourless. However, the DR0810 mutant was still red in colour and identical to the wild-type strain. Interestingly, the phenotype of the N-terminal motif (amino acids 19–69) deletion mutant ($\Delta crtI_{D60}$) was also colourless like $\Delta crtI$.

Subsequently, the carotenoid compositions of wild-type and mutants were analysed by HPLC. For the wild-type, more than 10 peaks were present in the elution profile of carotenoid extracts as detected at 470 nm (Fig. 2a). Peak 1 was identified as deinoxanthin, based on its retention time and absorption spectral features (Laurant *et al.*, 1997). No peak was detected in the elution profiles of $\Delta crtI$ by HPLC at 470 nm (Fig. 2b), indicating that most of the carotenoid

synthesis was blocked owing to the loss of DR0861. Moreover, as shown in Fig. 2(e), mutation of DR0861 resulted in the accumulation of phytoene, which is the substrate of phytoene desaturase. These results suggested that DR0861 might encode phytoene desaturase.

Complementation of $\Delta crtI$ with heterologous or homologous phytoene desaturase genes

Complementation *in vivo* was performed in order to confirm that the *crtI* gene is responsible for the loss of pigment in $\Delta crtI$. The complementary transformant exhibited red pigment when plasmid pRAD_{DR}, containing the intact DR0861 gene, was introduced into the colourless $\Delta crtI$ mutant. HPLC analysis revealed that its carotenoid composition was restored (Fig. 2c, f) and similar to that of the

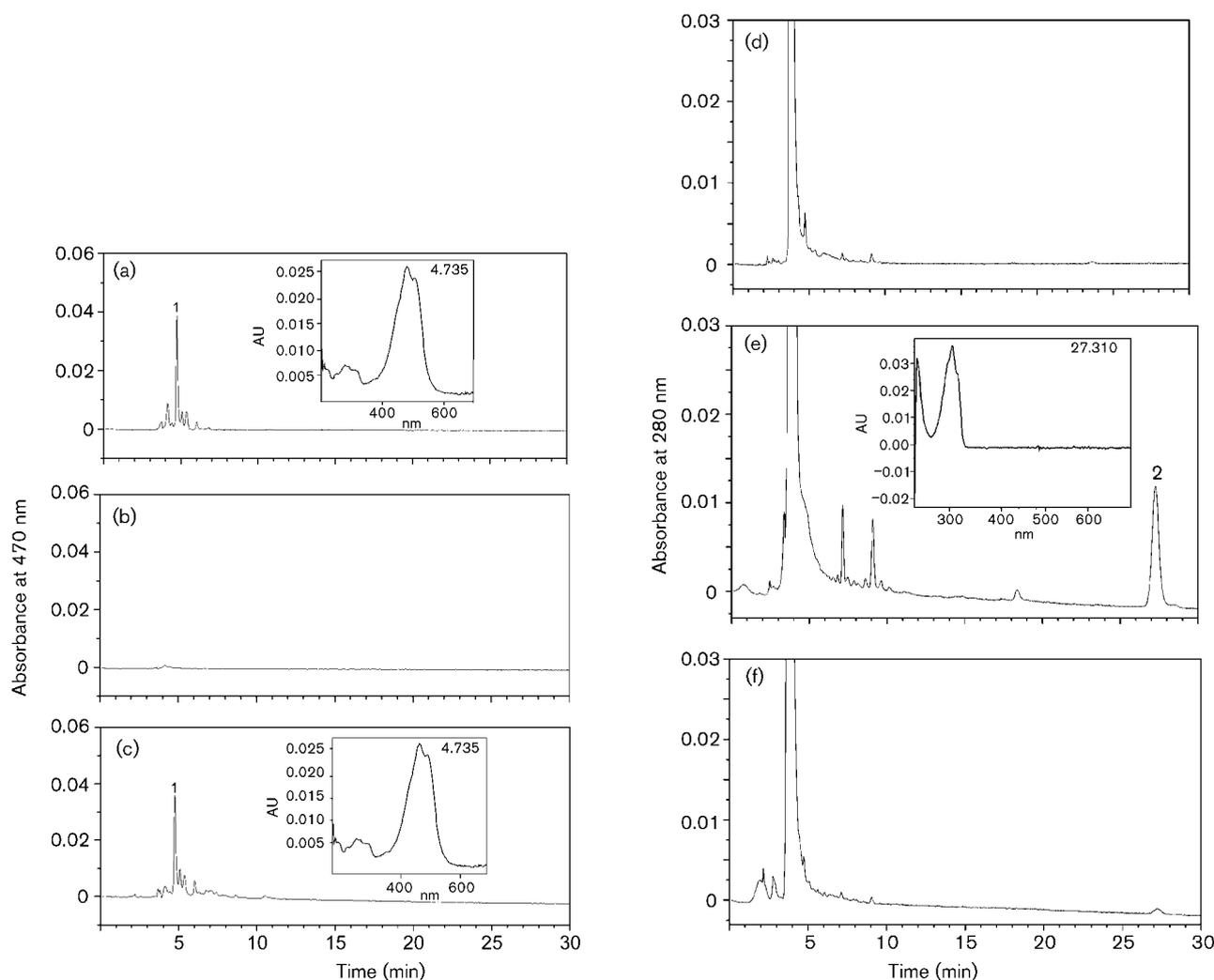


Fig. 2. HPLC analysis of carotenoids in *D. radiodurans*. (a–c) Detection at 470 nm: (a) wild-type, (b) DR0861 knockout mutant $\Delta crtI$, (c) DR0861 gene *in vivo* complementation strain. (d–f) Detection at 280 nm: (d) wild-type, (e) DR0861 knockout mutant $\Delta crtI$, (f) DR0861 gene *in vivo* complementation strain. Peak 1 was identified as deinoxanthin and peak 2 was phytoene. The peak at 5 min detected at 280 nm was identified as acetone. Absorption spectra for the major peaks are inset in the profiles.

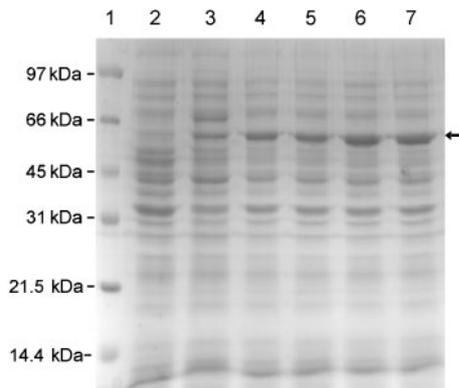


Fig. 3. SDS-PAGE analysis of proteins from *E. coli* strain BL21(DE3). Lane 1, molecular mass markers; lane 2, *E. coli* without pET28I plasmid; lane 3, *E. coli* with pET28I (without IPTG induction); lane 4, *E. coli* with induced expression of DR0861 gene (25 °C); lane 5, *E. coli* with induced expression of DR0861 gene (28 °C); lane 6, *E. coli* with induced expression of DR0861 gene (32 °C); lane 7, *E. coli* with induced expression of DR0861 gene (37 °C). The arrow indicates the location of the recombination-expressed DR0861 protein.

wild-type (Fig. 2a, d). Plasmid pRADI_{Eu} containing the *crtI* gene from *Er. uredovora* was also introduced into Δ crtI and it restored the carotenoid composition to that of the wild-type (data not shown).

Carotenoid composition in *E. coli* expressing DR0861

DR0861 was expressed in *E. coli* strain BL21. The protein extracted from cell homogenates was separated by 12% (w/v) SDS-PAGE as shown in Fig. 3. The expected protein expressed in *E. coli* had an apparent molecular mass of 60 kDa (lane 3–7), and was absent in the control strain (lane 2). Even when induced at different temperatures (25–37 °C), the recombinant protein was less than 10% (w/w) of the total cellular protein. This may be due to the low solubility of membrane-associated proteins in the cells or to biased codon usage (Fraser *et al.*, 1992; Matsumura *et al.*, 1997).

The carotenoid composition in *E. coli* containing different plasmids was measured by HPLC. The host strain carrying the plasmid pACCRT-EB remained colourless, and no lycopene was detected at 470 nm (Fig. 4a) and 15,15'-*cis*-phytoene was the only carotenoid as detected at 280 nm (Fig. 4c). When plasmid pET28I was introduced into *E. coli* with pACCRT-EB, the cell suspension showed red pigmentation after induction by IPTG. Lycopene accumulated as the major carotenoid and phytoene was scarcely detectable (Fig. 4b, d). This result indicated that DR0861 acts as phytoene desaturase (CrtI), which catalyses lycopene biosynthesis from phytoene.

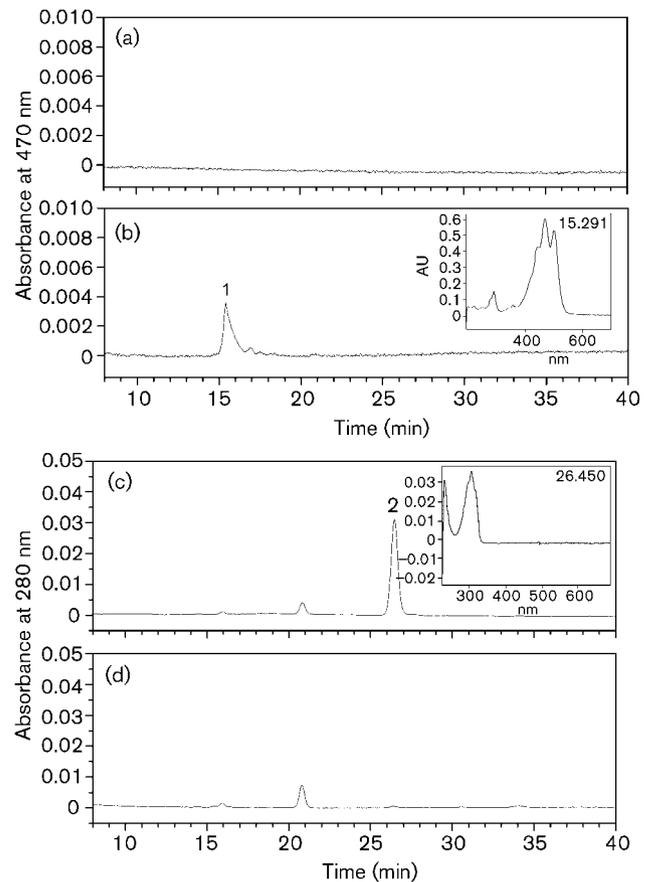


Fig. 4. HPLC analysis of carotenoids from *E. coli*. (a, b) Detection at 470 nm: (a) *E. coli* containing plasmid pACCRT-EB, (b) *E. coli* containing pACCRT-EB and pET28I (with IPTG). (c, d) Detection at 280 nm: (c) *E. coli* containing plasmid pACCRT-EB, (d) *E. coli* containing pACCRT-EB and pET28I (with IPTG). Peak 1, all-*trans*-lycopene. Peak 2, 15,15'-*cis*-phytoene. Absorption spectra for the major peaks are inset in the profile of panels (b) and (c).

In vitro phytoene desaturase activity assay

To further confirm that lycopene synthesis from phytoene in *D. radiodurans* was catalysed by DR0861 protein, the enzyme activity of DR0861 protein was assayed *in vitro*. Purified DR0861 protein is active enough to convert 15,15'-*cis*-phytoene to lycopene in sufficient quantities for detection by HPLC as shown in Fig. 5. The substrate phytoene was converted into lycopene as the end product of the desaturation reaction, and the peak typical of all-*trans*-lycopene (peak 1 in Fig. 5b) was found, with absorbance maximum at 470 nm, while 15,15'-*cis*-phytoene (peak 2 in Fig. 5a) had its absorbance maximum at 280 nm in the elution profile. From the *in vitro* and *in vivo* enzyme activity assays, we can conclude that lycopene in *D. radiodurans* is formed from phytoene through a desaturation reaction catalysed by DR0861 protein.

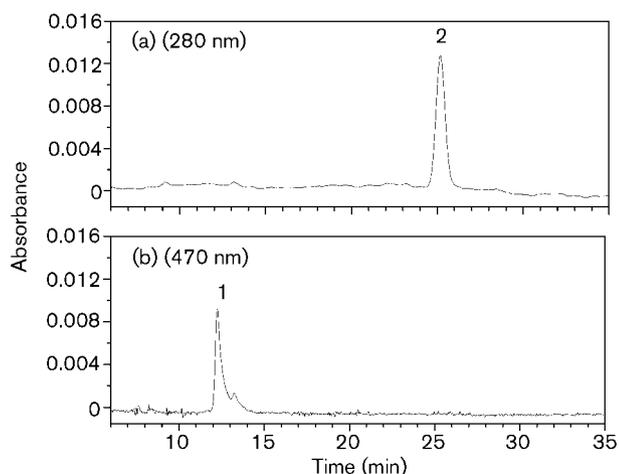


Fig. 5. HPLC analysis of carotenoid products in the *in vitro* reaction system. (a) Substrate of *in vitro* reaction detected at 280 nm. (b) Product of *in vitro* reaction detected at 470 nm. HPLC was carried out as described in Methods. The identified carotenoids are labelled 1 for all-*trans*-lycopene and its *cis* isomer, and 2 for 15,15'-*cis*-phytoene.

Effects of ionizing radiation and H₂O₂ on mutant Δ *cr*I

The cell survival of the wild-type strain and the mutants was evaluated after treatment with γ -rays. The wild-type strain exhibited a great resistance to irradiation with a D_{10} dose of 8554 Gy (Fig. 6), whereas the colourless mutant Δ *cr*I showed a significant decrease of radioresistance at doses higher than 2000 Gy ($P < 0.05$) and its D_{10} was 6803 Gy. This result indicated that loss of pigment affects the radioresistance of *D. radiodurans*. The survival rate of the Δ *cr*I_{DO} mutant was not significantly different from that of the *cr*I null mutant Δ *cr*I ($P > 0.05$). Moreover, when complemented with pRAD_{DR}, the ionizing radiation resistance of the mutants was completely restored. From these results, we deduced that carotenoids contribute to radioresistance and provide some protection in *D. radiodurans*. However, overexpression of the *cr*I gene in the wild-type hardly improved the extent of resistance (Fig. 6), presumably because the production of carotenoids is controlled by many carotenoid synthesis enzymes, precursor supply and product storage in the bacteria.

No distinct difference was observed between the wild-type and colourless mutants when they were cultivated with a low concentration of H₂O₂ (4.97 mM). However, significant differences were observed when cell cultures at stationary phase were incubated with H₂O₂ (10–40 mM) over a period of 1–3 h ($P < 0.05$). The survival rate of the colourless mutant was significantly decreased by treatment with 19.6 mM H₂O₂ for 30 min, while the red-pigmented R1 showed a greater resistance even after treatment for 3 h (Fig. 7a, b). A greater decrease in resistance was observed for the colourless strain compared to the wild-type

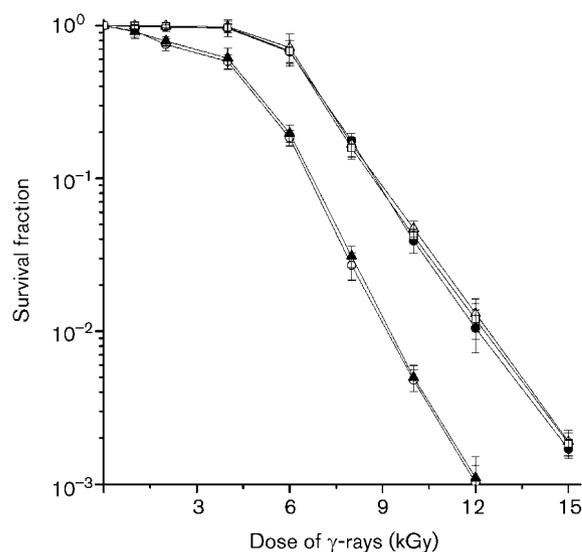


Fig. 6. Survival curves of *D. radiodurans* strains subjected to irradiation. Wild-type R1 (●), Δ *cr*I (○), Δ *cr*I_{DO} (▲), RI_O (△) and KI_{DR} (□). Values are the mean \pm SD of four independent experiments ($n = 12$).

($P < 0.01$) when H₂O₂ concentration exceeded 20 mM. Overexpression strain RI_O showed no increase in its survival rate when exposed to H₂O₂, compared with that of the wild-type under the same growth conditions (Fig. 7c).

Effects of lycopene accumulated in *E. coli* on cell survival

Lycopene acts as a strong antioxidant to scavenge reactive oxygen species (ROS) including singlet oxygen and hydroxyl radicals (Lowe *et al.*, 1999). To examine its protective effect against oxidative damage in *E. coli*, we expressed the *cr*I gene from *D. radiodurans* in *E. coli* containing pACCRT-EB and determined the amount of lycopene generated in the transformants, and investigated the survival rate of *E. coli* transformants exposed to different doses of H₂O₂ and γ -rays.

Table 3 shows the production of lycopene in various strains. Transformant BL_{EB} with pACCRT-EB showed a slight resistance to oxidative damage (Fig. 8a), due to the antioxidant effect of phytoene synthesized *in vivo* (Table 3). Without induction by IPTG, a small quantity of lycopene ($52.40 \pm 3.15 \mu\text{g g}^{-1}$) was produced in transformant BL_{EB}I (no IPTG). Compared to the control (*E. coli* host strain), the lycopene accumulated in *E. coli* BL_{EB} gave significant protection against H₂O₂ ($P < 0.001$). After induction by IPTG, the cells produced a large quantity of lycopene ($221.14 \pm 17.37 \mu\text{g g}^{-1}$) (Table 3). Overproduction of lycopene in the *E. coli* transformant had no influence on its survival in the absence of environmental stresses. However, a pro-oxidant phenomenon was observed in the *E. coli* after the transformant was exposed to H₂O₂. The cells

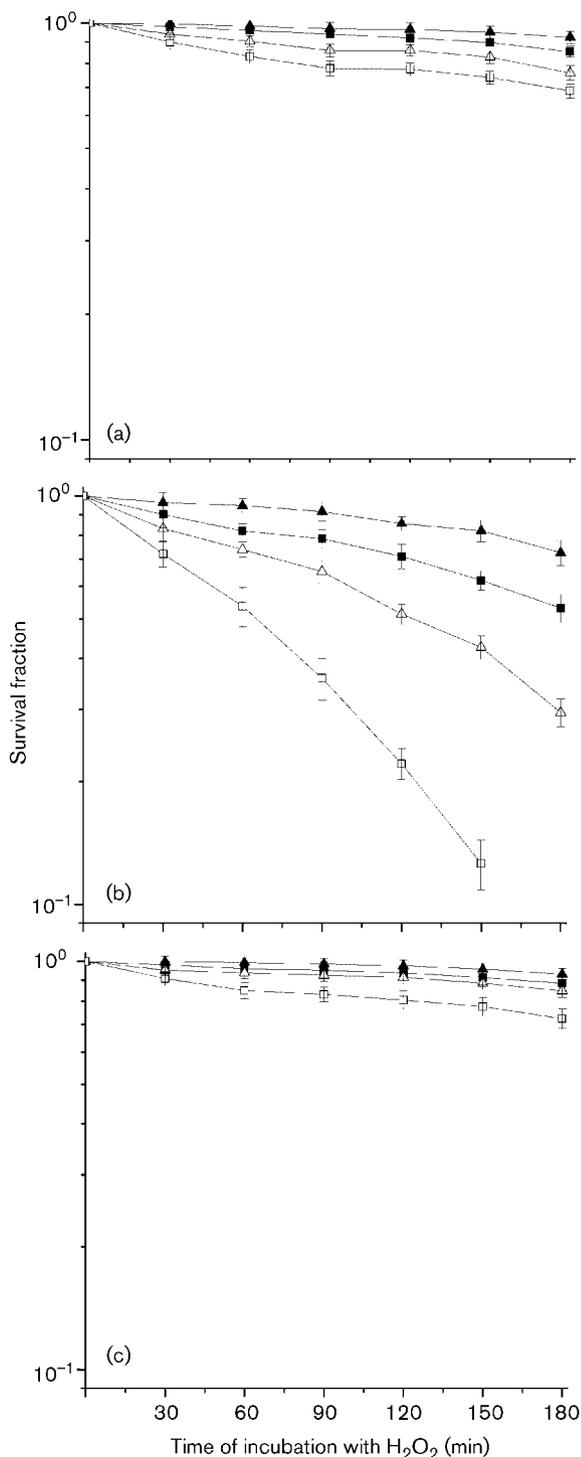


Fig. 7. Survival curves of *D. radiodurans* strains exposed to hydrogen peroxide. (a) Wild-type R1, (b) *crtI* knockout mutant Δ *crtI*, (c) *crtI* overexpressing strain R1_O. ▲, 9.01 mM H₂O₂; ■, 19.61 mM H₂O₂; △, 29.13 mM H₂O₂; □, 38.46 mM H₂O₂. Values are the mean \pm SD of four independent experiments ($n=12$).

overproducing lycopene became more sensitive to H₂O₂ than the wild-type, and lost most of their viability even when exposed to 1 mM H₂O₂ (Fig. 8a).

To further examine the role of carotenoids produced in *E. coli*, the transformants with different plasmids were exposed to γ -rays. Significant differences in survival rate between the colourless *E. coli* host strain and red-pigment transformant strains were observed. As shown in Fig. 8b, *E. coli* BL_{E_{BI}} without IPTG induction exhibited much greater radioresistance to the lethal effects of ionizing radiation than the control, *E. coli* BL21(DE3) ($P<0.01$). The D_{10} dose of the former was 175 Gy whereas that of the latter was 136 Gy. However, when lycopene was overproduced after induction by IPTG (Table 3), the survival rate of *E. coli* BL_{E_{BI}} exposed to γ -rays decreased significantly ($D_{10}=47.8$ Gy), compared to that of the control ($P<0.05$). This negative effect of overproduction of lycopene on cell survival is similar to the pro-oxidant effect of lycopene in the H₂O₂ treatment experiment.

DISCUSSION

D. radiodurans is famous for its remarkable resistance to ionizing radiation and oxidative stress (Mosely, 1983), and this extraordinary ability has been mostly attributed to its efficient DNA repair (Makarova *et al.*, 2001). Although some novel genes involved in DNA repair in *D. radiodurans* have been identified recently (Earl *et al.*, 2002; Hua *et al.*, 2003; Narumi *et al.*, 2004), the mechanisms underlying its extraordinary resistance have remained unclear. Furthermore, it was discovered that free radical scavengers had an important role in the protective mechanism of *D. radiodurans* (Daly *et al.*, 2004). The enzymic antioxidants including CAT and SOD act as important free radical quenchers and their mutants became more sensitive to ionizing radiation than the wild-type (Markillie *et al.*, 1999). Expression of PQQ synthase from *D. radiodurans* increased the radioprotective and oxidative stress protective capabilities of *E. coli* (Misra *et al.*, 2004). These findings suggest that enzymes and antioxidants contribute to the cellular defence system in *D. radiodurans*. Carotenoids are strong antioxidants that scavenge reactive oxygen species including singlet oxygen and hydroxyl radicals (Glaeser & Klug, 2005). The red-pigmented *D. radiodurans* synthesizes a unique carotenoid product that was identified as deinoxanthin. However, the deinoxanthin biosynthesis pathway and its functions in *D. radiodurans* have not been elucidated.

We determined that DR0861 encodes the bacterial-type phytoene desaturase (CrtI) in *D. radiodurans* on the basis of gene mutation, function complementation, heterologous expression and *in vitro* enzyme activity assays. From the sequence alignments and comparison analysis, DR0861 has the highest identity to the phytoene desaturase of *G. violaceus*, which was identified as the first oxygenic photosynthetic organism using a bacterial-type phytoene desaturase (CrtI) to convert phytoene into lycopene, rather than a cyanobacterial or plant-type phytoene desaturase (CrtP/Pds) (Steiger *et al.*, 2005; Tsuchiya *et al.*, 2005). The dinucleotide-binding motif and signature domains found

Table 3. Composition and content of carotenoids in *E. coli* transformants

Strain/description*	Carotenoid content [$\mu\text{g (g cell dry weight)}^{-1}$] [†]	
	Phytoene	Lycopene
BL _{EB} (no IPTG)	100 \pm 28.22	ND
BL _{EBI} (no IPTG)	2.52 \pm 0.28	52.40 \pm 3.15
BL _{EBI} (with IPTG)	0.84 \pm 0.06	221.14 \pm 17.37

*BL_{EB} (no IPTG): transformant strain BL_{EB} without IPTG induction; BL_{EBI} (no IPTG): transformant strain BL_{EBI} without IPTG induction; BL_{EBI} (with IPTG): transformant strain BL_{EBI} with IPTG induction.
[†]Values are means of three replicate determinations. ND, Not detected.

in some phytoene desaturases of bacteria and fungi (Armstrong *et al.*, 1989; Bartley *et al.*, 1990; Linden *et al.*, 1994; Ruiz-Hidalgo *et al.*, 1997) are also present in the sequence of DR0861 (Fig. 1). These observations indicate that phytoene desaturases including DR0861 existing in bacteria are conserved and may come from a common ancestor. Phylogenetic analysis based on *crtI* sequences provided strong support for this hypothesis (data not shown).

We found that DR0861 gene knockout and dinucleotide-binding domain deletion mutations inhibited lycopene synthesis and resulted in phytoene accumulation (Fig. 2b, e). Although the DR0810 gene, another putative phytoene desaturase gene annotated in the database, has more than 30% identity with the phytoene desaturase of *Rubrobacter xylanophilus*, our results showed that DR0810 was not involved in lycopene synthesis on the basis of gene knockout and HPLC analysis. Complementation of the knockout mutant with the *crtI* gene from *D. radiodurans* or *Er. uredovora* restored carotenoid synthesis in *D. radiodurans* completely (Fig. 2c), indicating that the inactive *crtI* gene was responsible for the loss of pigmentation in the Δ *crtI* mutant.

To investigate the protective role of carotenoids in *D. radiodurans*, we measured the cell survival rates of the colourless mutants and wild-type strain exposed to ionizing radiation and oxygen stress. The colourless mutants became significantly more sensitive to γ -rays and H₂O₂ than the wild-type. The sensitivity of colourless mutants was not as marked as that of DNA repair-defective strains. The resistance of the mutants could be recovered when the pigment was restored after the intact *crtI* gene from *D. radiodurans* or *Er. uredovora* was used for complementation. These results demonstrated that carotenoids provide some protection against radiation and oxygen damage in *D. radiodurans*. The relatively modest effect of carotenoids on cell survival may be due to the fact that the carotenoids are not involved in the DNA repair process directly, but participate in the protective mechanism of *D. radiodurans*

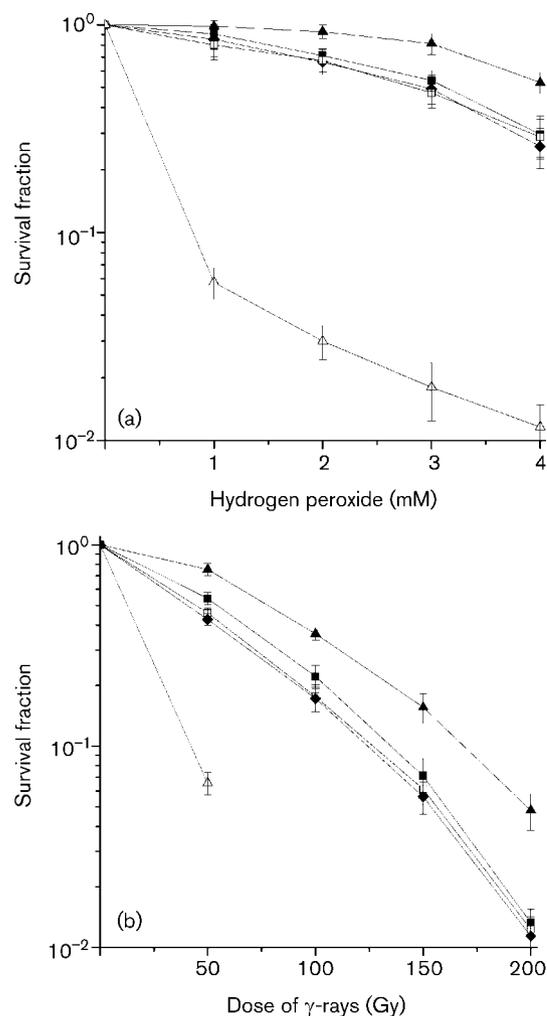


Fig. 8. Survival curves of *E. coli* transformants exposed to (a) hydrogen peroxide and (b) ionizing radiation. Strain BL21(DE3) was used as the wild-type and treated at stationary phase ($1\text{--}5 \times 10^{10}$ c.f.u. ml⁻¹). The treatment is described in detail in Methods. \square , BL21 (control); \blacksquare , BL21 carrying pACCRT-EB; \blacklozenge , BL21 carrying pET-28I (with IPTG induction); \blacktriangle , BL21 carrying pACCRT-EB and pET-28I (without IPTG induction); \triangle , BL21 carrying pACCRT-EB and pET-28I (with IPTG induction). Values are the mean \pm SD of four independent experiments ($n=12$).

as ROS scavengers. The carotenoids can quench harmful ROS generated from water radiolysis to prevent oxidative damage to proteins, including DNA repair proteins, and membrane lipid peroxidation. It has been reported that carotenoids, including lycopene, can efficiently inhibit membrane lipid peroxidation (Stahl *et al.*, 1998). Pyrrolo-quinoline quinone, another antioxidant metabolite in this bacterium, has also shown obvious protective effects on the cell (Khairnar *et al.*, 2003). It is not clear whether there is a synergetic effect between this compound and the carotenoids. Further research needs to be done to elucidate the protection mechanism of carotenoids and the relationships of these antioxidant metabolites in *D. radiodurans*.

Furthermore, our results showed that overexpression of the *crtI* gene in *D. radiodurans* did not result in a notable increase in the carotenoid content and the cells showed no markedly enhanced resistance to γ -rays and H_2O_2 (Fig. 6, 7). This was probably due to carotenoid synthesis being controlled by many carotenoid synthesis enzymes, precursor supply and product storage in the bacteria.

To confirm the function of the DR0861 gene, it was expressed in *E. coli* containing pACCRT-EB. The transformant became red-pigmented and accumulated lycopene (Fig. 4b). Desaturation activity of DR0861 protein was further tested by *in vitro* enzyme activity assays. The substrate 15,15'-*cis*-phytoene was converted into all-*trans*-lycopene as the end product of the desaturation reaction (Fig. 5). These results indicated that DR0861 had desaturase activity, which was responsible for the conversion of phytoene to lycopene through four-step, not three-step, desaturation in *D. radiodurans*. We also determined the amount of lycopene synthesized in *E. coli* transformants, and assessed its protective effects on *E. coli*. Without IPTG induction, the production of lycopene in *E. coli* transformants was less than that in IPTG-induced transformants, and led to a significant enhancement of resistance to H_2O_2 and γ -rays, suggesting that carotenoid synthesis can supply *E. coli* cells with some protection. However, after induction with IPTG, the superfluous production of lycopene had a pro-oxidant effect when the cells were exposed to H_2O_2 or γ -rays, which caused a significant decrease in cell survival rate (Fig. 8). On the other hand, the survival capacity of *E. coli* containing only pACCRT-EB or pET28I receiving the same treatment with IPTG was not affected. Pro-oxidant actions of lycopene have also been reported in other biological systems (Lowe *et al.*, 1999; Yeh & Hu, 2000). The antioxidant activity of some carotenoids may shift into pro-oxidant activity, depending on their concentrations as well as on the biological environment including oxygen tension (Palozza, 1998). Therefore, the pro-oxidant effect of lycopene in this study may result from its high levels in the *E. coli* transformants and the oxidative stress from the treatment with H_2O_2 and γ -rays.

In this study, we identified a typical *crtI*-type phytoene desaturase gene (DR0861) involved in the deinoxanthin biosynthesis pathway of *D. radiodurans* and investigated the protective effects of carotenoids against radiation and oxidative damage in *D. radiodurans* and *E. coli*. Further investigation of the remaining unidentified carotenogenic enzyme genes and the function of the corresponding carotenoid metabolites would facilitate elucidation of carotenoid biosynthesis pathways and actions of carotenoid metabolites in *D. radiodurans*.

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