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Metabolic Engineering of *Deinococcus radiodurans* for the Production of Phytoene

Sun-Wook Jeong†, Chang Keun Kang†, and Yong Jun Choi*

School of Environmental Engineering, University of Seoul, Seoul 02504, Republic of Korea

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**Corresponding author*
Phone: +82-2-6490-2873;
Fax: +82-2-6490-2859;
E-mail: yongjun2165@uos.ac.kr

†These authors contributed equally to this work.

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A metabolically-engineered *Deinococcus radiodurans* R1 strain capable of producing phytoene, a colorless C_{40} carotenoid and a promising antioxidant, has been developed. To make this base strain, first, the *crtI* gene encoding phytoene desaturase was deleted to block the conversion of phytoene to other carotenoids such as lycopene and γ-carotene. This engineered strain produced 0.413 ± 0.023 mg/l of phytoene from 10 g/l of fructose. Further enhanced production of phytoene up to 4.46 ± 0.19 mg/l was achieved by overexpressing the *crtB* gene encoding phytoene synthase and the *dxs* genes encoding 1-deoxy-D-xylulose-5-phosphate synthase gene, and by deleting the *crtD* gene. High cell-density culture of our final engineered strain allowed production of 10.3 ± 0.85 mg/l of phytoene with the yield and productivity of 1.04 ± 0.05 mg/g and 0.143 ± 0.012 mg/l/h, respectively, from 10 g/l of fructose. Furthermore, the antioxidant potential of phytoene produced by the final engineered strain was confirmed by in vitro DPPH radical-scavenging assay.

Keywords: Metabolic engineering, *Deinococcus radiodurans*, phytoene, antioxidation

Introduction

Isoprenoids, a diverse class of natural organic molecules derived from isoprene, are important industrial compounds that have been widely used in various industrial products such as food additives, pharmaceuticals, biofuels, and cosmetic ingredients. Especially because of their excellent antioxidant activity, some isoprenoids such as β-carotene, lycopene, and lutein are getting much attention for their potential use as natural pharmaceutical ingredients. Thus, the size of the global market for antioxidant isoprenoids has been gradually increasing over the last few decades.

Phytoene ($C_{40}H_{64}$), one of the colorless carotenoids, is an intermediated metabolite of the isoprenoid biosynthetic pathway as well as a starting metabolite for the biosynthesis of various carotenoids [1]. Despite the lack of pigment for dissipating the oxidative energy of singlet oxygen, it shares the inherent beneficial efficacies of naturally occurring carotenoids such as anti-oxidation, anti-inflammation, and immune system-boosting ability. Previous studies on the effectiveness of phytoene have shown that proliferation of human prostate cancer and mammary cells are significantly inhibited by the regular ingestion of phytoene-containing phytonutrients [1–4]. Moreover, considering the critical weaknesses of various carotenoids, such as limited availability and light sensitivity resulting from the distinctive color that makes carotenoids somewhat unfavorable in cosmetic applications, it also has great advantages over other colored carotenoids in terms of scalability and availability [5]. With this increasing interest in phytoene and the rapidly increasing market for carotenoids has come a greater need for large-scale fermentative production of phytoene through metabolic engineering of microorganisms.

There have been several reports on the production of phytoene by metabolic engineering of the isoprenoid biosynthetic pathway. Recently, metabolically-engineered *Thermococcus kodakarensis* strain expressing Saci_1734 from *Sulfolobus acidocaldarius* capable of producing up to 0.75 mg/l of phytoene was reported [6]. Moreover, 3.4-fold increased phytoene production was achieved by disruption of acetyl-CoA synthetase I gene followed by double-overexpression of Saci_1734. More recently, genetically-engineered *Dunaliella salina* V-101 capable of producing phytoene up to 108.34 mg/ 100 mg DCW was developed

by down-regulation of phytoene desaturase using RNAi and antisense methods [5]. Another study also reported the highest level of phytoene production (up to 10 mg/g DCW) using *Xanthophyllomyces dendrorhous* by deleting the *crtI* gene together with overexpressing the *crtE* and *crtYB* genes [7, 8].

An extremophilic bacterium, *Deinococcus radiodurans* has been well-known for its inherent biosynthetic pathway for the biosynthesis of various carotenoids [9, 10]. It also has an extremely high concentration of intracellular glucose-6-phosphate and NADP(H), which are essential for the synthesis of carotenoids [11]. Moreover, the bacterium has various genes of particular interest that deserve to be engineered for increasing tolerance to external stresses [9]. Significantly enhanced cellular tolerance to external stresses such as osmotic pressure, high salts, temperature, and pH that can be caused during fermentation was observed by heterologous overexpression of those of genes [12, 13].

Thus, *D. radiodurans* is of particular interest due to its potential to be used as a platform strain and its superiority to other microorganisms such as *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* for the production of a variety of carotenoids. However, there has been no report about intensive engineering of *D. radiodurans* for industrial application. This situation seems due to the lack of genetic manipulation tools for the engineering of *D. radiodurans*. Although lots of available metabolic information on carotenoid pathway together with genetic engineering tools have been developed [10, 14–18], the metabolic engineering of *D. radiodurans* for the production of high-value carotenoid compounds has not yet been reported. These observations led us to explore the potential of *D. radiodurans* for the production of phytoene by rational metabolic engineering for the first time.

To achieve this goal, the *crtI* and *crtD* genes encoding phytoene synthase and carotenoid 3′,4′-desaturase, respectively, were sequentially deleted in *D. radiodurans*. Then, the *crtB* and *dxs* genes were overexpressed to increase the carbon flux towards phytoene. Furthermore, we also evaluated the antioxidant activity of the produced phytoene by in vitro DPPH radical-scavenging assay.

Materials and Methods

Bacterial Strains and Cultivation

All bacterial strains and plasmids in this study are listed in Table 1. *Deinococcus radiodurans* strains were routinely cultured at 30°C in TGY (0.5% tryptone, 0.3% yeast extract, and 0.1% glucose). Batch culture was carried out using modified minimal media (per liter) containing 20 mM phosphate buffer (pH 7.0), 15 mM $(NH_4)_2SO_4$, 5 μM $MnCl_2$, 0.8 mM $MgCl_2$, 0.18 mM $CaCl_2$, 10 g fructose, 10 mg/ml vitamins mix (Sigma), 50 mg/l L-cysteine, 25 mg/l L-histidine, 25 mg/l L-methionine, and 1 g yeast extract [19]. To obtain high cell density, *D. radiodurans* were cultured in modified rich medium (50 mM HEPES, 10 g/l tryptone, 5 g/l yeast extract, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and 5 μM $MnCl_2$) supplemented with 10 g/l fructose [21]. The cell density was measured using a spectrophotometer

Table 1. Bacterial strains and plasmids used in this study.

	Description	Reference
Strains		
D. radiodurans		
R1	Wild type (ATCC13939)	ATCC
E. coli		
DH5α	Host for plasmid subclones	Lab stock
Plasmids		
pAM1	A derivative of pKatAPH3 containing *lox66-km^r-lox71* cassette	
pAM2	A derivative of p13840 containing P_{GroES}-*cre*-P_{GroES}-*cm^r*	[12]
pRADZ3	*E. coli-D. radiodurans* shuttle vector for overexpression of genes controlled by *groE* promoter	
pAM41	A derivative of pRADZ3 containing *crtB* gene	This study
pAM42	A derivative of pRADZ3 containing *crtE* gene	This study
pAM73	A derivative of pRADZ3 containing *dxs* gene	This study
pAM74	A derivative of pRADZ3 containing *idi* gene	This study
pAM103	A derivative of pAM41 containing *crtE* gene	This study
pAM104	A derivative of pAM41 containing *dxs* gene	This study
pAM105	A derivative of pAM41 containing *idi* gene	This study

(Thermoscientific, USA) with a wavelength of 600 nm and converted into DCW (g/l) using a standard curve. *Escherichia coli* DH5α was used as the cloning host for propagation of expression plasmid and cultured at 37°C in Luria-Bertani medium (LB). When necessary, antibiotics were added at final concentration of 100 μg/ml ampicillin for *E. coli*, and 3 μg/ml chloramphenicol and 25 μg/ml kanamycin for *D. radiodurans*.

Knockout Mutant Construction

The construction of all knockout mutants in this study was carried out using Cre-*loxP* system as previously described [14]. In brief, the DNA fragment containing *lox66-km*^{r}*-lox71* cassette flanked by approximately 1 kb of both up and downstream nucleotide sequences of target genes (*crtI*, *dr0810*, or *crtD*) was amplified by PCR from pAM1 plasmid and *D. radiodurans* genomic DNA using appropriate primer sets. To fuse each PCR fragment, fusion PCR was performed using *pfu* DNA polymerase kit (AccuPower Pfu PreMix, Bioneer), according to the manufacturer's instruction. The resulting PCR fragments were introduced into 50 μl of *D. radiodurans* cells. The mixtures were incubated for 12 h at 32°C without agitation and spread onto 2X TGY agar containing 25 μg/ml of kanamycin to select a recombinant. The antibiotic maker integrated in *D. radiodurans* genome was removed by introducing plasmid pAM2, which harbors Cre recombinase. A recombinant *D. radiodurans* harboring pAM2 was cultured at 30°C for 12 h without selection pressure and further incubated at 37°C during 12 h for plasmid curing. After removing antibiotic markers and plasmids, the DNA sequencing and diagnostic PCR were performed to verify the deletion of the target genes. The primers used in this study are listed in Table 2.

DNA Manipulation and Plasmid Constructions

A NucleoSpin kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and a MegaQuick-Spin plus fragment DNA purification kit (Intron biotechnology, Korea) were employed for purification of plasmids and PCR products, respectively. The restriction enzymes were purchased from Enzynomics (Korea).

For the construction of plasmids expressing, the *crtB* , *crtE*, *dxs*, and *idi* genes were amplified using corresponding primer sets (Table 2), and cloned into pRADZ3 at SpeI and NotI sites make the pAM41, pAM42, pAM73, and pAM74, respectively. To construct pAM103, pAM104, and pAM105 expressing the *crtE*, *dxs* or *idi* genes together with the *crtB* gene, the *crtE*, *dxs* or *idi* genes were amplified using GroE-F and CrtE-R1, Dxs-R1, or Idi-R1 primers, respectively, from pAM42, pAM73, or pAM74 plasmids as templates and subsequently cloned into pAM41, behind the *crtB* gene, respectively, using NotI and BamHI sites.

Analytical Methods

The carotenoid extraction procedure was carried out as described previously with slight modification [21]. Fresh colonies were cultured overnight at 30°C in 5 ml TGY broth and then subcultured in 50 ml defined minimal media (or rich media) supplemented with 1% fructose and 0.1% yeast extract. After incubation for 72 h, 10 ml of cells were harvested and washed three times with

Table 2. Primers used in this study.

Primer	Sequence (5′ to 3′)[a]
crtI knockout	
crtI-1	catagtgaaagaaacgtctg
crtI-2	agcttatcgataccgtcgacgacagtaaacctcggaagtc
crtI-3	gacttccgaggtttactgtcgtcgacggtatcgataagct
crtI-4	ttaagcggaatccgtatgaccatgcctgcaggtcgactct
crtI-5	agagtcgacctgcaggcatggtcatacggattccgcttaa
crtI-6	ttggtccacatgctggtgca
dr0810 knockout	
dr0810-1	gttgtatacgctcgacggcg
dr0810-2	agcttatcgataccgtcgacactccacaggcgacttcaca
dr0810-3	tgtgaagtcgcctgtggagtgtcgacggtatcgataagct
dr0810-4	gtataagacccagcaacccgcatgcctgcaggtcgactct
dr0810-5	agagtcgacctgcaggcatgcgggttgctggctcttatac
dr0810-6	ctgaccaacgcctacagcgt
crtD knockout	
crtD-1	catgttcatcaccagccgca
crtD-2	agcttatcgataccgtcgactgtagcgggagcagtatcac
crtD-3	gtgatactgctcccgctacagtcgacggtatcgataagct
crtD-4	aatgccttctcgccatccagcatgcctgcaggtcgactct
crtD-5	agagtcgacctgcaggcatgctggatggcgagaaggcatt
crtD-6	gatgtcgcgagttcgaatct
crtB expression	
crtBF	aagtactagtatgaggtctagggccggtt
crtBR	ctatgcggccgctcagccgtggaccgcgccca
crtE expression	
crtEF	aagtactagtatgcgtcccgaactgctcgc
crtER	ataggcggccgctcacttctcccgcgtcgcca
dxs expression	
dxsF	ctagactagtgtgaacgaacttcccggcac
dxsR	taacgcggccgcctacacctcaatcggcacgt
idi expression	
idiF	gttaactagtatgcggctggacactgtgtt
idiR	ctaggcggccgccttgcagaggggtcccttta
Multiple gene expression	
groE-F	cgtggcggccgctcggcttggaagcacgtatt
crtE-R	gtatgaatcctcacttctcccgcgtcgcca
dxs-R	gttaggatcctacacctcaatcggcacgt
idi-R	ttgaggatccttgcagaggggtcccttta

[a]Underlines indicate restriction enzyme sites.

sterilized water. Carotenoids from the cell pellet were extracted with 3 ml acetone/methanol solvent (7:2 (v/v)). The extracts were kept at −80°C before use. The phytoene standard (15-cis-phytoene) was obtained from Toronto Research Chemical (Cat. No. P398805, North York, Canada) and used for quantitative analysis. Standard sample was dissolved in acetone and prepared for standard calibration curve ranging from 1 to 100 mg/l. Carotenoids in the extracts were analyzed by HPLC using an Agilent 1260 Infinity (Agilent technologies, USA) equipped with quaternary pump system chromatograph coupled with a variable wavelength detector (VWD). A C18 column (ZORBAX Eclipse XDB-C18, 4.6 × 250 mm, 5 μm) was used and eluted with a mixture of methanol, acetonitrile, and isopropanol (50:40:10 (v/v)) at a flow rate of 2 ml/min. Phytoene was detected by monitoring absorbance at 280 nm. LC/MS detection system (Agilent 6120 Quadrupole LC/MS, Agilent technologies, USA) was used to determine the molecular mass of phytoene from the cell extracts. Mass spectra were measured between *m/z* 450 and 650 in positive ionization mode (ESI^+) at a scan rate of 0.79 sec/cycle. The operating conditions of LC/MS were as follows: capillary voltage = 4000; drying gas flow = 10 l/min (N_2); nebulizer pressure = 50 psig; drying gas temperature = 300°C. Carbohydrate contents from the culture media were analyzed using MetaCarb 87H column (250 × 4.6 mm, Agilent) with 0.005 N H_2SO_4. The products were detected using a refractive index detector (RID). The flow rate was 0.5 ml/min at 30°C.

DPPH Assay

The free radical scavenging assay was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) to estimate the antioxidant activity of phytoene. Samples were mixed with 1.5 μl of DPPH and incubated at room temperature for 30 min in the dark. Ascorbic acid was used as reference sample (0.1 mg/ml). The reaction samples were measured at 515 nm using a UV spectro-

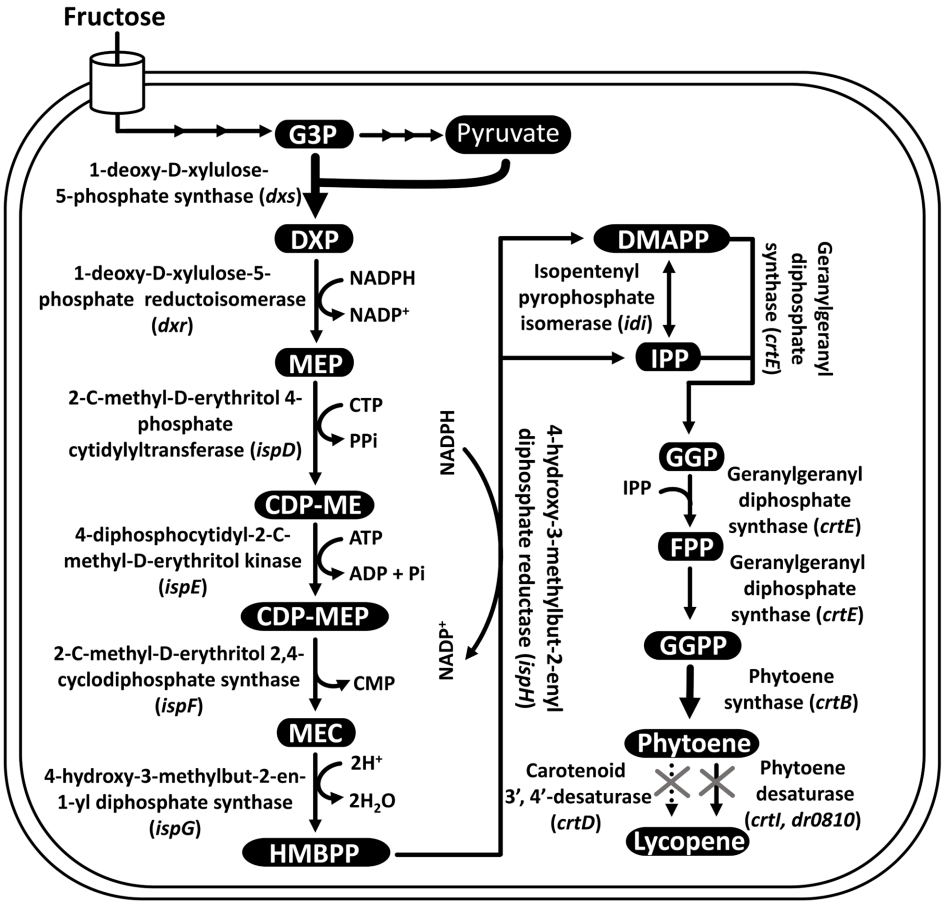

Fig. 1. Scheme of carotenogenesis pathway and metabolic engineering strategies for phytoene production in *D. radiodurans*.
The gray X's denote gene that is knocked out. Thick arrows indicate increased fluxes by directly overexpressing the corresponding genes. Intermediates in the metabolic pathway: G3P, glyceraldehyde 3-phosphate; DXP, 1-Deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; CDP-MEP, 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; MEC, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

photometer. The scavenging activity was calculated using an equation as described previously [22].

Results and Discussion

Construction of *crtI*-deleted *D. radiodurans* as a Base Strain

To construct a base strain capable of producing phytoene, rational metabolic engineering of the carotenoid biosynthetic pathway was performed as shown in Fig. 1. The 1-Deoxy-D-xylulose 5-phosphate (DXP) is first generated by condensation of glyceraldehyde 3-phosphate (G3P) and pyruvate. DXP is converted to 2-C-methyl-D-erythritol 4-phosphate (MEP), which is spontaneously converted to (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP) through sequential conversion of metabolites such as 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-MEP), and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEC). The HMBPP is converted to geranyl diphosphate (GPP), which is also then sequentially converted to farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), and finally to phytoene (Fig. 1). In the carotenoid biosynthetic pathway, phytoene is converted to lycopene by phytoene desaturase encoded by the *crtI* gene. Thus, deletion of the *crtI* gene encoding phytoene desaturase was performed to make the base strain block the conversion of phytoene to lycopene as the first step. The resulting Δ*crtI D. radiodurans* strain was able to produce 0.413 ± 0.023 mg/l of phytoene, whereas wild-type strain could not produce phytoene at all (Figs. 2A and 2B). The LC/MS analysis demonstrated the molecular ions at *m/z* 545.5 ($[M+H]^+$), 567.6 ($[M+Na]^+$), and 583.5 ($[M+K]^+$), which was identical to that of phytoene standard and mass value of phytoene (Fig. 2C). Thus, the deletion of the *crtI* gene in *D. radiodurans* was to be functional to make a base strain for microbial production of phytoene.

Previously, putative phytoene desaturase encoded by the *dr0810* gene was also found to be involved in conversion of

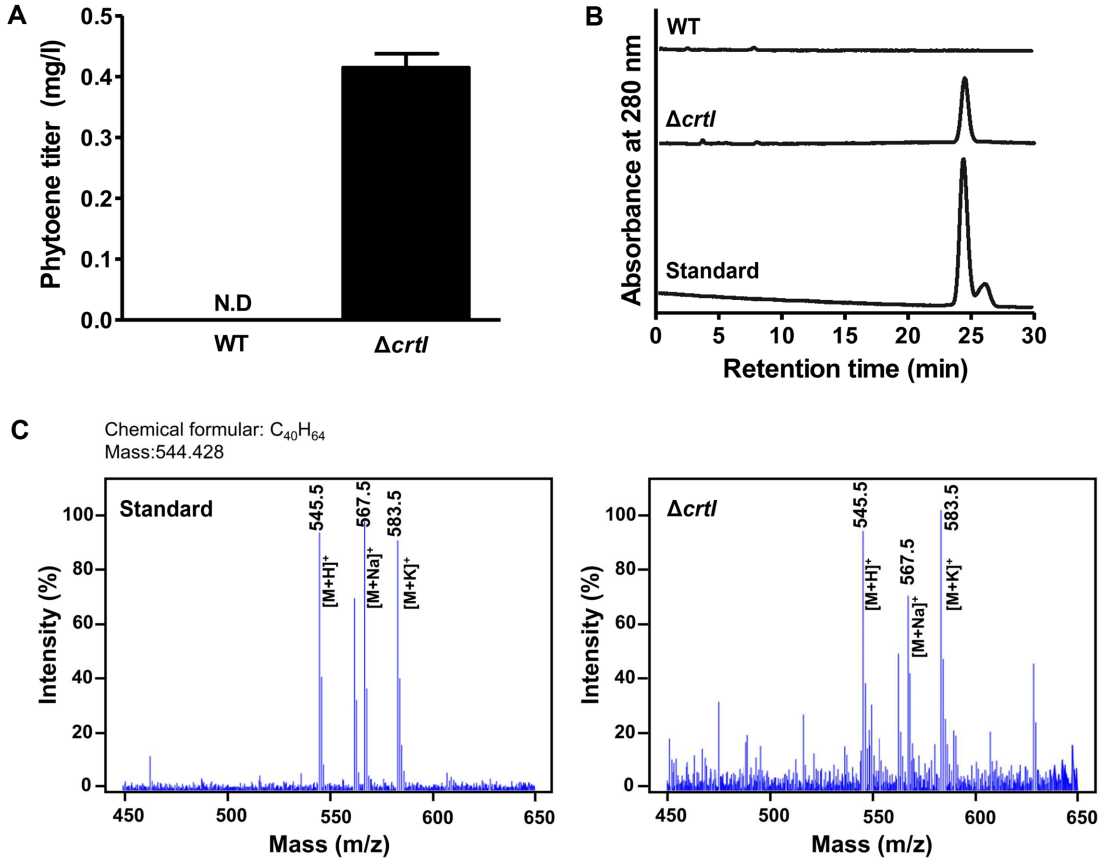

Fig. 2. Identification of microbial phytoene produced by *D. radiodurans*.
(**A**) The effect of deletion of the *crtI* gene on phytoene production. (**B**) HPLC analysis of phytoene extracted from Δ*crtI D. radiodurans*. (**C**) LC-MS analysis of phytoene produced by Δ*crtI D. radiodurans*. ND: not detected. All experiments were performed in triplicate; error bars denote standard deviations.

phytoene to lycopene [9, 10]. Thus, the effect of putative phytoene desaturase on phytoene production was further investigated by deleting the *dr0810* gene in Δ*crtI D. radiodurans* strain. However, no significant changes of phytoene titer were observed in Δ*crtI*Δ*dr0810* mutant strain (0.41 ± 0.016 mg/l). This result was coincidence with the previous study reporting the function of CrtI that has crucial role for conversion of phytoene to lycopene rather than *dr0801* in *D. radiodurans* [10].

Increasing the Carotenoid Pools by Overexpression of the *dxs* and *crtB* Genes

There had been several reports describing the enhancement of desired target products by the identification of rate-limiting steps and engineering to support metabolic bottlenecks in the carotenoid biosynthetic pathway. Previously, the *dxs* gene encoding D-1-deoxyxylulose 5-phosphate synthase has been proved as the first rate-limiting step on the MEP pathway so that overall isoprenoid pools were dramatically increased by overexpression of the *dxs* gene [21–23]. As another rate-liming step, the inter-conversion of DMAPP with IPP, which is mediated by isopentenyl pyrophosphate isomerase encoded by the *idi* gene is also one of the critical metabolic bottlenecks that need engineering so as to increase isoprenoid pools [26, 27]. To examine the effect of increased carbon flux towards rate-limited and immediate metabolites on phytoene production, the *dxs*, *idi*, *crtE* (encoding geranylgeranyl diphosphate synthase), or *crtB* (encoding phytoene synthase) genes were overexpressed in Δ*crtI D. radiodurans* strain. Among the four different candidates, the Δ*crtI D. radiodurans* expressing the *crtB* gene was found to be the best for phytoene production (up to 1.85 ± 0.18 mg/l). Interestingly, unlike previous studies, no significant effects were observed in the other strains expressing *crtE* (0.38 ± 0.09 mg/l), *dxs* (0.48 ± 0.05 mg/l), or *idi* (0.47 ± 0.07 mg/l) compared to Δ*crtI D. radiodurans* strain (0.34 ± 0.1 mg/l) (Fig. 3A). Moreover, as can be seen in Fig. 3B, more than 70% increased phytoene production (up to 3.25 ± 0.21 mg/l) was achieved by additional overexpression of the *dxs* gene in Δ*crtI*

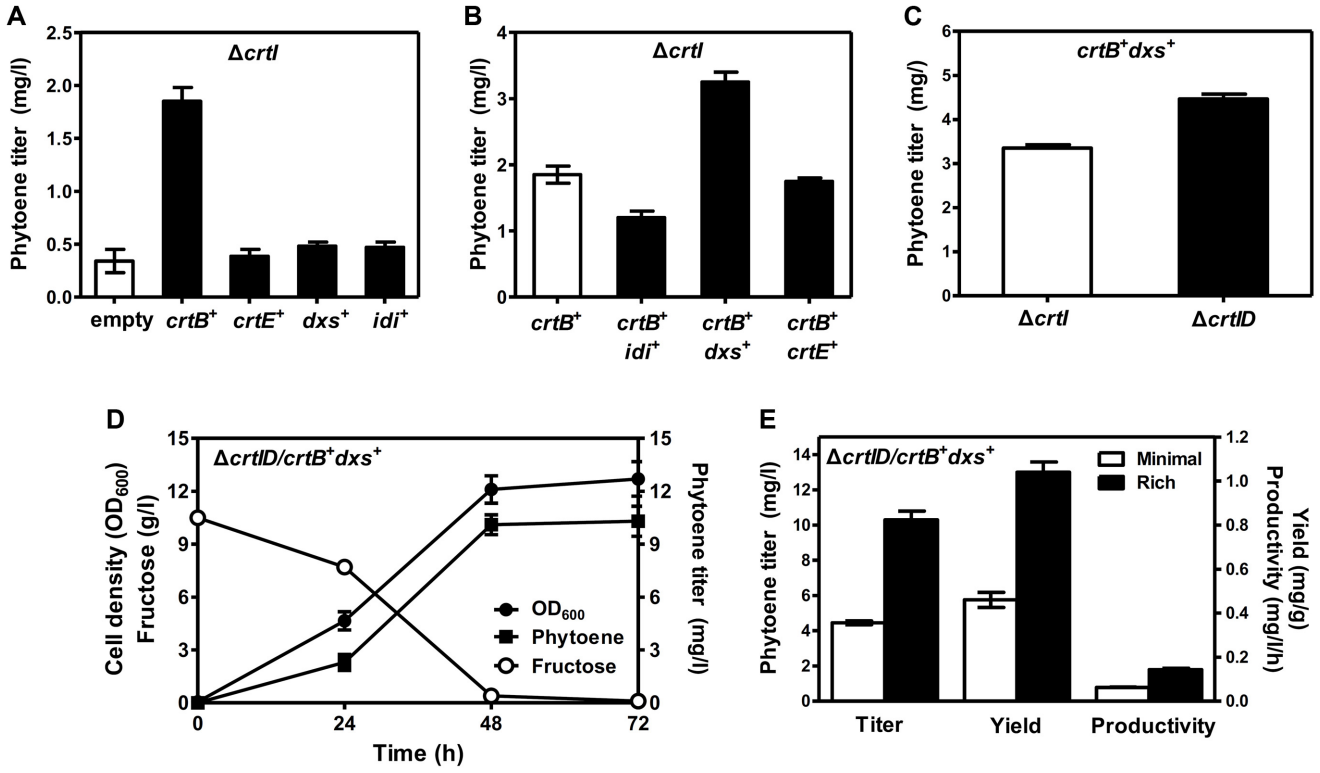

Fig. 3. Enhanced production of phytoene by stepwise engineering of *D. radiodurans*.

(**A**, **B**) The effect of overexpressing genes involved in carotenoid biosynthetic pathway on phytoene production in Δ*crtI* mutants. (**C**) The effect of deletion of the *crtD* gene on phytoene production. (**D**) Time-course profiles of the Δ*crtID* mutants expressing the *crtB* and *dxs* genes in modified rich medium during 72 h. (**E**) Comparative analysis of the phytoene titer, yield, and productivity of final engineered strain cultured in minimal and modified rich media. All experiments were performed in triplicate; error bars denote standard deviations.

D. radiodurans strain expressing the *crtB* gene, whereas phytoene titer was slightly decreased in the *crtB* expressing Δ*crtI D. radiodurans* strain together with the *idi* gene (1.2 ± 0.14 mg/l), or *crtE* gene (1.5 ± 0.07 mg/l). It was interesting to note that the effects of compensation for rate-limiting steps were somewhat different from previous reports. As the *idi* gene was also suggested to be responsible for rate-limiting steps in the isoprenoid pathway, the enhancement of phytoene production was indeed predictable in this study. This discordance might be because of the different enzymatic capacity and carbon flux according to their metabolic characteristics.

Enhanced Production of Phytoene by Deletion of the *crtD* Gene

Previously, Tian *et al*. [16] reported that carotenoid 3′, 4′-desaturase, which is encoded by the *crtD* gene has 21% of homology with phytoene desaturase (CrtI) that is responsible for dehydrogenation of phytoene to lycopene, and displays C-3′, 4′-desaturation activity of monocyclic carotenoid in *D. radiodurans*. Another study also reported the enhanced production of lycopene through the upregulation of genes involved in the isoprenoid pathway by the deletion of the *crtD* gene in *Haloarcula japonica* strain [28]. Thus, the Δ*crtID* mutant strain was constructed by deleting the *crtD* gene in Δ*crtI D. radiodurans* to check the effect of deletion of the *crtD* gene on phytoene production. As we expected, the Δ*crtID D. radiodurans* expressing the *crtB* and *dxs* genes was able to produce 25% increased phytoene up to 4.46 ± 0.19 mg/l (Fig. 3C) with the yield of 0.46 ± 0.06 mg/g and productivity of 0.062 ± 0.003 mg/l/h from 10 g/l of fructose (Fig. 3C). In spite of a natural carotenoid-producing strain, the titer is still less than that of previous studies reporting phytoene production using eukaryotic cells [5, 8, 29] and non-natural producers [6, 30]. We hypothesized that this is mainly because of low cell density. Previously, the maximum cell density of *D. radiodurans* up to 42.2 at OD_{600} was achieved by cultivation in modified rich medium [20]. Therefore, to investigate if this low titer is indeed due to the low cell density, high-cell density culture was performed in modified rich medium. When the final engineered strain was cultured in modified rich medium, as we expected, not only did cell density increase by 2.3-fold (from 5.3 to 12.7), but phytoene production also increased 2.8-fold (from 4.46 ± 0.19 to 10.3 ± 0.85 mg/l) compared with that obtained in minimal medium from 10 g/l of fructose (Fig. 3D). Moreover, the yield and productivity of phytoene were increased by 2.26-fold (from 0.46 ± 0.06 to 1.04 ± 0.05 mg/g) and by 2.3-fold (from 0.062 ± 0.003 to 0.143 ± 0.012 mg/l/h), respectively (Fig. 3E).

Antioxidant Activity of Phytoene

Due to their potent radical scavenging activity, various carotenoids such as lycopene, astaxanthin and β-carotene have been widely used in cosmetic ingredients, food additives, and therapeutic agents. Although a few studies describing microbial production of phytoene have been reported, the antioxidant potential of microbial phytoene has not yet been investigated. Therefore, to evaluate the performance of the radical scavenging activity of phytoene produced by final engineered *D. radiodurans* strain, in vitro DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging assay was performed based on previous methods [22, 31, 32]. As can be seen in Fig. 4, the DPPH scavenging activity was dramatically increased in a phytoene dose dependent manner and significantly enhanced activity (89.1% ± 0.28%) was observed at 5 mg/ml of phytoene within 30 min (Fig. 4).

We have developed genetically engineered a *D. radiodurans* strain capable of producing phytoene for the first time. This was successfully achieved by rational metabolic engineering such as deletion of competitive pathway and overexpression of key genes. The final engineered *D. radiodurans* strain was able to produce 10.3 ± 0.85 mg/l of phytoene from 10 g/l of fructose. Furthermore, potent antioxidant performance was observed in phytoene produced by the recombinant *D. radiodurans* strain we developed. Despite the efficient

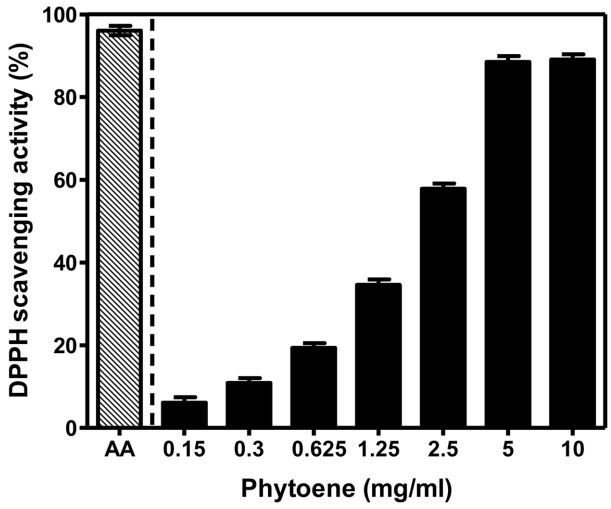

Fig. 4. Antioxidant effects of phytoene analyzed by in vitro DPPH radical-scavenging assay.

All experiments were performed in triplicate; AA, ascorbic acid. Error bars denote standard deviations.

microbial production of phytoene, further intensive strain development through systems metabolic engineering strategies such as 1) optimizing metabolic pathway to reinforce carbon flux towards phytoene, 2) engineering of key enzymes to overcome hurdle reactions, and 3) in silico simulation for identifying metabolic flux networks, should be carried out to enhance performance for the production of phytoene. In conclusion, the final recombinant *D. radiodurans* strain we developed is a promising strain for the efficient production of phytoene worthy for use as a potent antioxidant.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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