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Laboratory-Scale Production of ^{13}C-Labeled Lycopene and Phytoene by Bioengineered *Escherichia coli*

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ABSTRACT: Consumption of tomato products has been associated with decreased risks of chronic diseases such as cardiovascular disease and cancer, and therefore the biological functions of tomato carotenoids such as lycopene, phytoene, and phytofluene are being investigated. To study the absorption, distribution, metabolism, and excretion of these carotenoids, a bioengineered *Escherichia coli* model was evaluated for laboratory-scale production of stable isotope-labeled carotenoids. Carotenoid biosynthetic genes from *Enterobacter agglomerans* were introduced into the BL21Star(DE3) strain to yield lycopene. Over 96% of accumulated lycopene was in the *all-trans* form, and the molecules were highly enriched with ^{13}C by ^{13}C-glucose dosing. In addition, error-prone PCR was used to disrupt phytoene desaturase (*crtI*) function and create a phytoene-accumulating strain, which was also found to maintain the transcription of phytoene synthase (*crtB*). Phytoene molecules were also highly enriched with ^{13}C when the ^{13}C-glucose was the only carbon source. The development of this production model will provide carotenoid researchers a source of labeled tracer materials to further investigate the metabolism and biological functions of these carotenoids.

KEYWORDS: tomato, lycopene, phytoene, ^{13}C, *E. coli*

INTRODUCTION

Tomato consumption has been correlated with decreased risks of chronic diseases including prostate cancer and cardiovascular disease.[1] Among many nutrients and phytochemicals in fresh tomatoes and tomato products, carotenoids are thought to be the bioactive components responsible for disease prevention. Many epidemiological studies, clinical trials, animal studies, and in vitro studies have focused on the most abundant tomato carotenoid, lycopene.[2] However, the absorption, distribution, metabolism, and excretion (ADME) of phytoene and phytofluene, the precursors of lycopene and the second and third most abundant tomato carotenoids, have not been fully studied.

As it is difficult to completely deplete fat-soluble carotenoids from humans, stable isotope-labeled carotenoid tracers allow a single carotenoid dose to be followed within pre-existing endogenous pools. Previously, labeled lutein, β-carotene, or vitamin A has been utilized to provide information regarding (1) the fraction of the ingested amount that is absorbed (bioavailability), (2) the fraction of that which is converted to vitamin A in the body (bioconversion), (3) the estimated body stores of vitamin A, and (4) the interaction between retinal and rhodopsin.[3−8] To date, no tracer studies utilizing isotope-labeled phytoene or phytofluene have been conducted, either in animals or in humans, and therefore very little is known regarding the ADME. Producing labeled phytoene and phytofluene has been a challenge to answer these important biomedical questions.

Currently, most of the commercially pure carotenoids are produced by chemical synthesis, but a few biotechnological production systems including microalgae, fungi, tomato cell culture, and *Escherichia coli* culture are being developed and optimized. Several concepts for the phototrophic mass culture of algae have been proposed and compared.[9−11] Among many fungal strains, *Blakeslea trispora* has been employed for the production of natural lycopene, commercialized by Vitatene. In addition, cell suspension cultures of tomato (*Lycopersicon esculentum* cv. VFNT cherry) have also been developed for laboratory-scale production of phytoene, phytofluene, and lycopene in our laboratory.[12] This system was further optimized by treating cells with two bleaching herbicides, 2-(4-chlorophenylthio)-triethylamine (CPTA) and norflurazon, and utilization of high carotenoid producing cell lines.[13,14]

It has been well-known that *E. coli* harboring carotenogenic genes from a Gram-negative, nonphotosynthetic bacterium, *Enterobacter agglomerans* (or *Pantoea agglomerans*, formally *Erwinia herbicola*), can accumulate carotenoids (Figure 1). This carotenogenic gene cluster was first cloned from *E. agglomerans* in 1986,[15] and the DNA sequence was reported in 1990.[16] Since then, novel carotenoids have been produced in *E. coli* by combining different carotenoid biosynthetic genes from *E. agglomerans* and other species.[17−20] Additionally, carotenoid production was tremendously improved by introducing the mevalonate pathway,[21] increasing precursor supply,[22] or knocking out unnecessary genes in *E. coli*.[23−25] Because *E. coli* can utilize glucose as the sole carbon source, and ^{13}C-glucose is a readily available labeled source material, this system could be used to produce isotopically labeled carotenoids. Therefore, the goal of this research was to utilize carotenoid-producing *E. coli* for

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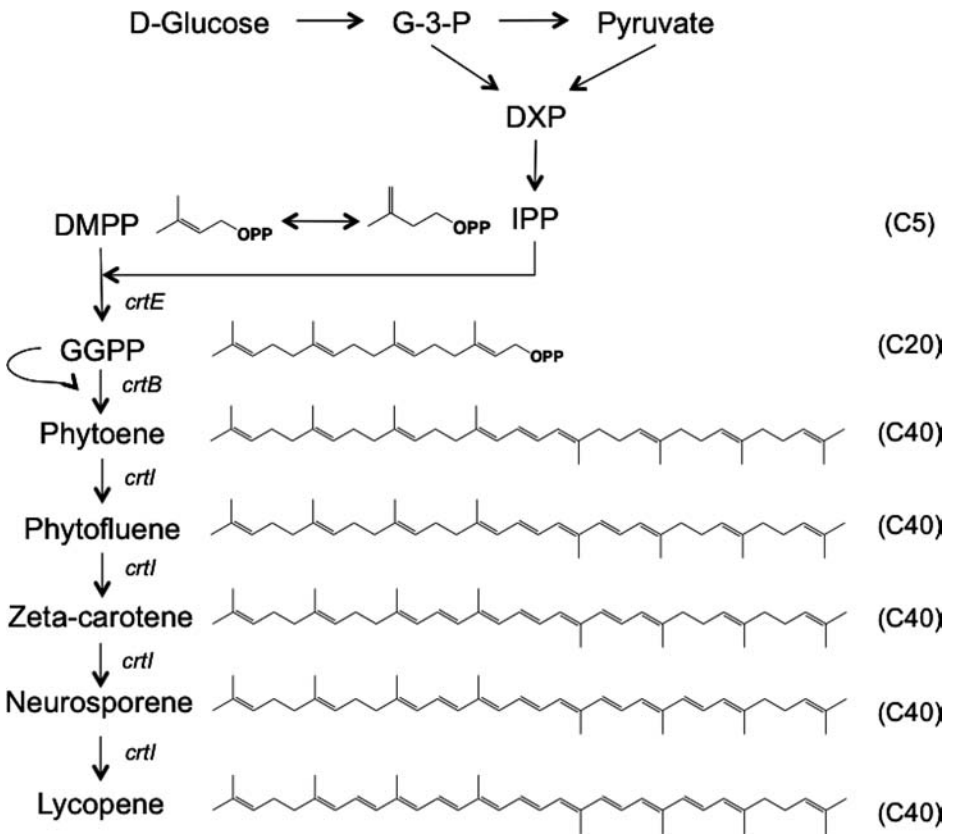

Figure 1. Carotenoid synthesis pathway in *E. coli* harboring pAC-LYC, which contains GGPP synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*).

producing stable isotope-labeled tomato carotenoids, lycopene and phytoene, for future biomedical research.

MATERIALS AND METHODS

Chemicals and Reagents. All bacterial strains and culture media were purchased from Invitrogen (Carlsbad, CA), and primers for PCR were synthesized by Integrated DNA Technologies (Coralville, IA). All restriction enzymes were purchased from New England Biolabs (NEB, Ipswich, MA), and molecular biology kits were purchased from Qiagen (Valencia, CA). All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO), and all organic solvents were of high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA).

Construction of Truncated Plasmid pAC-PTE-TR. The plasmid pAC-LYC,[26] which contains GGPP synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*) from *E. agglomerans*, was digested by restriction enzymes *Sbf* I and *Bgl*II (NEB), and the digested products were separated on a semipreparative 0.8% agarose gel. The larger DNA fragment, containing the pACYC-184 backbone, was retrieved with a Qiagen Gel Extraction Kit, blunted by a NEB Quick Blunting Kit and ligated by a NEB Quick Ligation Kit. This truncated pAC-LYC, named pAC-PTE-TR hereafter, was amplified by *E. coli* strain DH5α and confirmed with electrophoresis and DNA sequencing by an ABI 3730XL capillary sequencer at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana−Champaign.

Construction of Mutated Plasmid pAC-PTE-MT. GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to create the mutant library. The gene *crtI* was amplified by sense primer 5′-TGACATATGAAAAAAACCGTTGTGATTGGCG-CAGGC-3′ and antisense primer 5′-ATTCTCGAGTTATCAGGCT GGCGGTGGCTTTCG-3′ per the manufacturer's instructions for high-error-rate PCR. The PCR products were then purified by a Qiagen PCR Purification Kit and mixed with pAC-LYC. The mixture was digested by restriction enzymes *Sbf*I and *Bgl*II, purified by Qiagen PCR Purification Kit, ligated by NEB Quick Ligation Kit, and transformed into *E. coli* strain DH5α. The transformants were grown on LB agar plates with the antibiotic chloramphenicol (34 μg/mL), and white colonies were amplified and screened by HPLC for phytoene production. Plasmid pAC-PTE-MT was isolated by a Qiagen Miniprep Kit, and the selected mutated-*crtI* was sequenced.

Bacterial Culture in LB Broth. *E. coli* strain BL21Star(DE3) and the transformants were grown and maintained in LB broth supplemented with 34 μg/mL chloramphenicol at 31 °C and 200 rpm. Overnight cultures were used to inoculate 5 mL cultures with a ratio of 1:100, and the cells were grown at 31 °C and 200 rpm for 12, 18, 24, and 48 h. Cells were harvested by 3220*g* centrifugation at 4 °C for 10 min, washed with 1× cold phosphate-buffered saline (PBS), weighed, and frozen at −80 °C until further analysis.

Bacterial Culture in 2XM9 Minimal Salts Media. For carotenoid accumulation and labeling experiments, BL21Star(DE3) and the transformants were grown in 2XM9 media, which contained 5 g/L unlabeled or [U]-^{13}C-glucose, 34 μg/mL chloramphenicol, and double the amount of salts in 1XM9 media, except for $CaCl_2$ and $MgSO_4$. Overnight cultures from LB were used to inoculate 5 mL cultures with a ratio of 1:100, and the cells were grown at 31 °C and 200 rpm for 12, 18, 24, and 48 h. LB broth was removed by centrifugation before cells were inoculated in 2XM9. Cells were harvested by 3220*g* centrifugation at 4 °C for 10 min, washed with 1× cold PBS, weighed, and frozen at −80 °C until further analysis.

Analysis of Glucose and Acetate by HPLC. Glucose and acetate concentrations of culture media were determined by HPLC

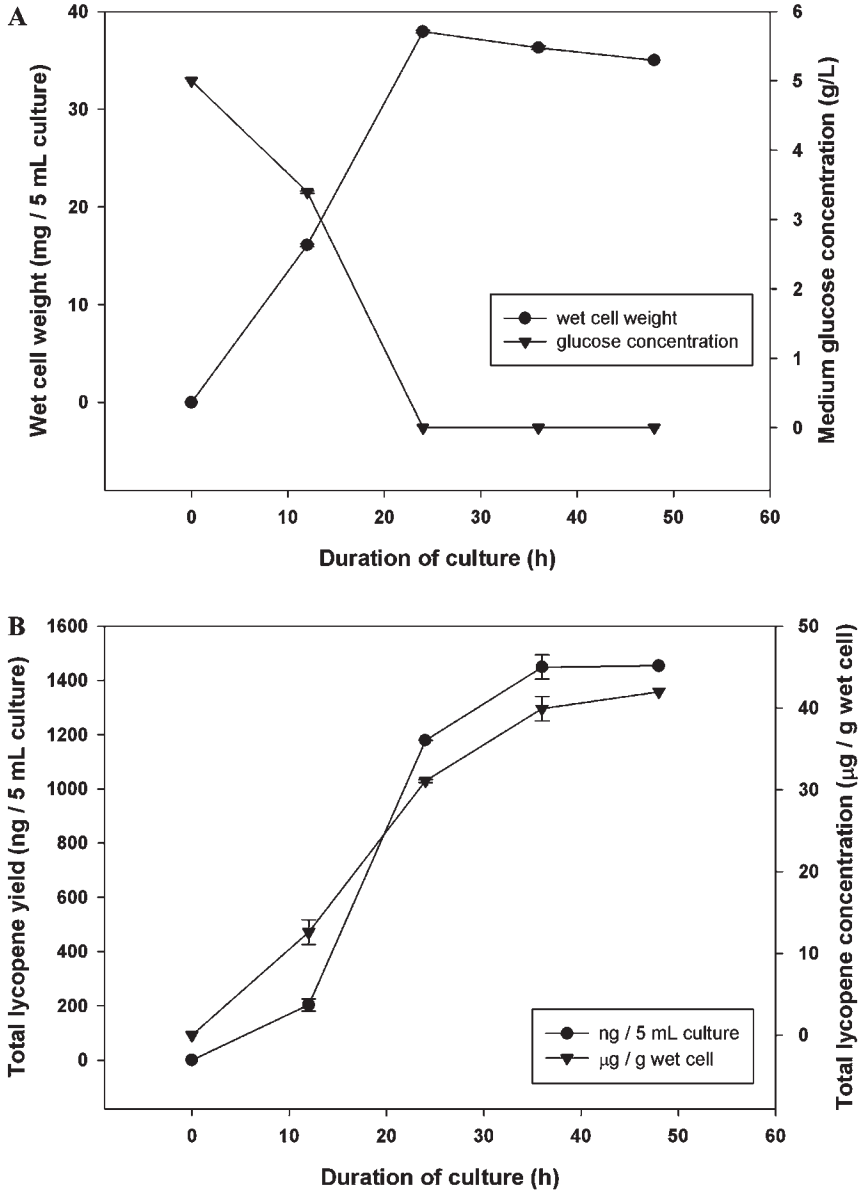

Figure 2. Characteristics of *E. coli* BL21Star(DE3) harboring pAC-LYC: (A) cell growth and the corresponding glucose concentration in 2XM9 media; (B) accumulation of total lycopene. Data are presented as the mean ± SE with $n = 3$.

(Agilent Technologies 1200 series) equipped with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) kept at 50 °C. The mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.6 mL/min. The culture medium was injected into the system without further purification, and the quantification of glucose was calculated on the basis of a standard curve.

Carotenoid Extraction. Bacterial cell pellets were thawed on ice, suspended in 50 μL of deionized water, and briefly vortexed to achieve homogeneous suspensions. Acetone (500 μL) was then added and followed immediately by a 10 s vortex period and 5 min water bath sonication. A total of 200 ng of *all-trans*-β-carotene (DSM, Basel, Switzerland) was added as an internal standard, because the system did not contain any of this cyclic carotenoid. The liquid and solid phases were then separated by 4 °C centrifugation at 16000*g* for 10 min. The acetone/water phase was transferred to clean centrifuge tubes, evaporated by argon, and partitioned by 500 μL of hexanes/methyl *tert*-butyl ether (MTBE) (1:1, v/v) two times. The extracts were combined and dried under argon. The samples were stored at −20 °C for no more than 24 h before HPLC analysis, with the entire extraction process performed under yellow light.

Analysis of Carotenoids by HPLC. The HPLC system is composed of two pumps (model SD-200, Rainin Dynamax, Walnut Creek, CA), a YMC Carotenoid C30 column (4.6 × 250 mm, 3 μm) with a guard column, an 18 °C column cooler, and a photodiode array detector (model 2996, Waters, Milford, MA). The mobile phases A (methanol/15% ammonium acetate aqueous solution = 98:2, v/v) and B (methanol/MTBE/15% ammonium acetate aqueous solution = 8:90:2, v/v) were used, with the following gradient profile: 0 min, 0% B; 30 min, 100% B; 40 min, 100% B; 45 min, 0% B; 50 min, 0% B. Samples were reconstituted in MTBE for injection, and phytoene was measured at 286 nm. Phytoene standards (BASF, Ludwigshafen, Germany) were prepared in petroleum ether using extinction coefficient $A^{1\%}_{1\ cm} = 1250$ at 286 nm. Lycopene standards (DSM) were prepared in hexane using extinction coefficient $A^{1\%}_{1\ cm} = 3450$ at 472 nm, and *all-trans*-β-carotene

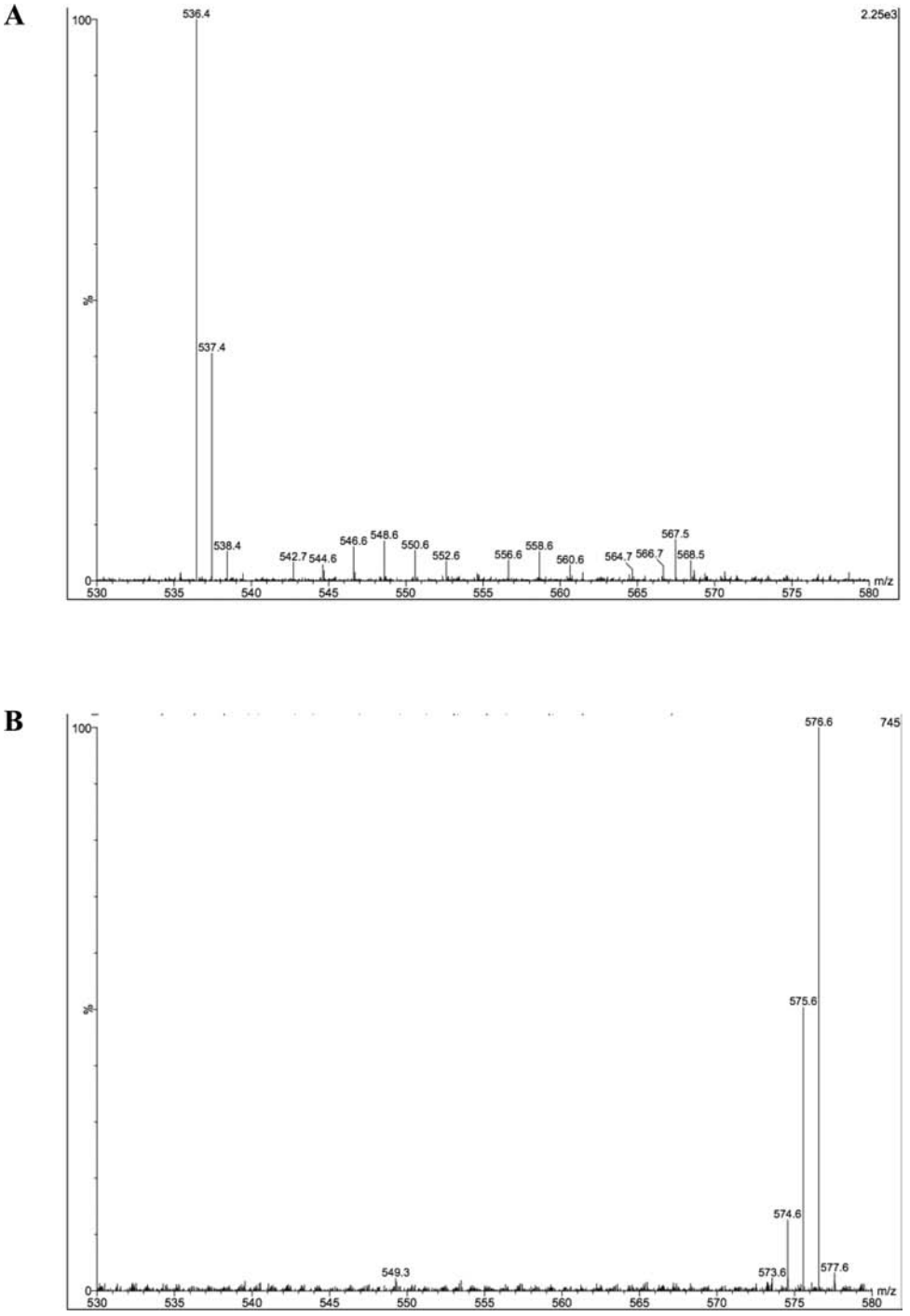

Figure 3. Isotopomer distribution of (A) ^{12}C-*all-trans*-lycopene ($^{12}C_{40}H_{56}$, MW = 536.4) and (B) ^{13}C-labeled *all-trans*-lycopene ($^{13}C_{40}H_{56}$, MW = 576.4), measured by ESI positive mode in a Q-ToF mass spectrometer.

standards (DSM) were prepared in *n*-hexane using extinction coefficient $A^{1\%}_{1\ cm}$ = 2592 at 452 nm.

Mass Spectrometry. Mass spectrometry was performed by the Mass Spectrometry Center in the School of Chemical Sciences at the University of Illinois at Urbana−Champaign. Unlabeled and labeled samples were purified by HPLC separation and completely dried before sample submission. The lycopene samples were dissolved in acetone and analyzed by Micromass Q-ToF Ultra (Waters) with electrospray ionization positive mode (ESI+) and high-resolution scanning. The phytoene samples were dissolved in anisole[27] and analyzed by a ThermoFinnigan LCQ Deca XP (Thermo Scientific, West Palm Beach, FL) with atmospheric pressure chemical ionization positive mode (APCI+) and low-resolution scanning.

RNA Extraction and Quantitative Real-Time PCR of *crtE* and *crtB*. Total RNA was extracted from cells using Trizol (Invitrogen) per the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). mRNA expression of selected genes was measured via real-time PCR using SYBR Green Master Mix (Applied Biosystems). Reactions were monitored by an ABI Prism 7900HT. Primer pairs were

Figure 4. Comparison of plasmid constructs used in this study: (A) phytoene desaturase (*crtI*) operons modified by truncation or mutation; (B) alignment of protein sequences of wild-type *crtI* and mutated *crtI*.

designed to measure GGDP synthase (*crtE*) (sense, 5′-GTCAGCCCACTACCCACAAAA-3′; antisense, 5′-GCGGCGATCAGACCAAAG-3′), phytoene synthase (*crtB*) (sense, 5′-GATGAGGGGGTGGTGGAT-3′; antisense, 5′-CAATAGCCGCATCGTCAATAATAT-3′), and 16s (*rrsB*) (sense, 5′-GCATAACGTCGCAAGACCAA-3′; antisense, 5′-GCCGTTACCCCACCTACTAGCT-3′). A validation experiment was performed on each set of primers to confirm efficiency and product specificity. A serial dilution was used to create a standard curve for quantification, and 16s was used as a housekeeping gene.

Statistical Analysis. Microsoft Excel for Mac 2011 was used to perform Student's *t* test. Figures were produced by SigmaPlot 2001 (Systat Software Inc., San Jose, CA).

RESULTS AND DISCUSSION

Lycopene Accumulation in BL21Star(DE3) Harboring pAC-LYC. The *E. coli* strain BL21Star(DE3) harboring the plasmid pAC-LYC, created by Cunningham et al.,[26] was used for lycopene production. Prior to the labeling run, *E. coli* was maintained in LB agar or LB broth. For the labeling run, 2XM9 minimal medium was used, which contained doubled nitrogen and [U]-^{13}C-glucose as the only carbon source to maintain growth characteristics of *E. coli* that were similar to the culture in LB broth. Initially, ^{12}C-glucose was provided in the 2XM9 minimal medium at the 5 g/L concentration to observe lycopene accumulation; glucose was completely depleted by 24 h (Figure 2A). Over 60% of glucose was used between 12 and 24 h, which suggested that the cells were in the logarithmic growth phase, which was further confirmed by measuring the OD_{600} (data not shown) and the wet cell weight (Figure 2A). Wet cell weight reached its maximum at 24 h, and a trace amount of acetate accumulated in the medium at 24 h and was depleted by 48 h.

Lycopene concentration was also measured during the 48 h culturing period (Figure 2B). From a 5 mL culture, a total of 1.4 μg of lycopene was obtained, 14, 71, and 15% of which were produced during 0−12, 12−24, and 24−36 h, respectively. Compared with the glucose concentration in the medium, *E. coli* seemed to continue producing lycopene even though the glucose had been depleted. The metabolic intermediates, acetate or pyruvate, which supported the lycopene production, would potentially be utilized by *E. coli* during 24−36 h. Interestingly, during the course of culture, the percentage of *all-trans*-lycopene increased over time and reached >96% by 24 h (data not shown), which is representative of its level of abundance in tomatoes. Less than 4% of total lycopene was 9-cis and 13-cis, and no other lycopene isomers were detected. Trace amounts of phytoene were observable only from the 12 h samples, indicating that the phytoene desaturase from *E. agglomerans* was highly efficient in catalyzing the desaturation reaction, which led to the accumulation of the major product, lycopene.

Isotopomer Distribution of ^{13}C-Labeled Lycopene. High-resolution mass spectrometry was used to observe the isotopomer distributions of unlabeled and labeled lycopene (Figure 3). HPLC-purified material was ionized by electrospray ionization and analyzed by a quadrupole mass sector and a time-of-flight mass sector. Under the positive mode of ionization, it was clear

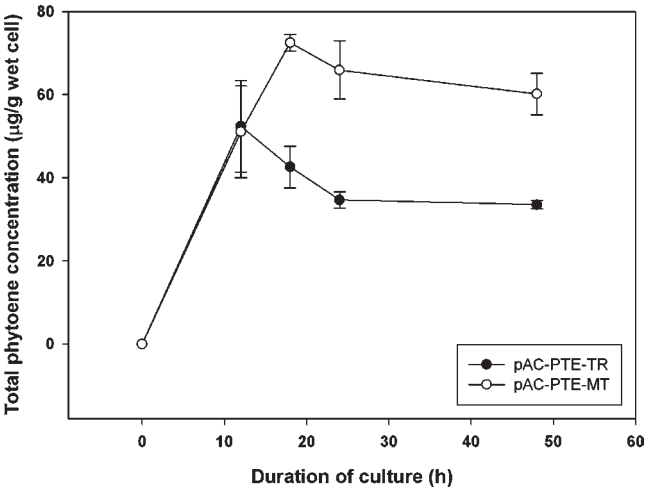

Figure 5. Phytoene accumulation in *E. coli* BL21Star(DE3) harboring pAC-PTE-TR and pAC-PTE-MT, cultured in LB broth. Data are presented as the mean ± SD with $n = 2$.

that the ion m/z 536.4 [$M^{\bullet}$] was the majority of the unlabeled lycopene, followed by the ions m/z 537.4 and 538.4 and a few other ions ranging between m/z 546.6 and 568.5. Because the natural abundance of ^{13}C is 1.109%, it is expected that a few lycopene molecules would naturally contain some ^{13}C, which resulted in the observation of ions with a higher m/z ratio. Comparing the mass spectra of labeled lycopene with unlabeled lycopene, interestingly, the majority of ions are m/z 576.6 [$M + 40^{\bullet}$], followed by the ions m/z 575.6 [$M + 39^{\bullet}$] and 574.6 [$M + 38^{\bullet}$], which indicates that the biosynthesized lycopene was mostly built from the [U]-^{13}C-glucose and is highly enriched with ^{13}C (Figure 3).

Error-Prone PCR Rescued Phytoene Production by Maintaining *crtB* Transcription. In the *E. agglomerans* carotenogenic gene cluster, there are two promoter regions reported by To et al.[28] One of the promoters was located within the gene *crtE*, and another was located within the gene *crtI*. Therefore, deletion of *crtI* could result in the disruption of *crtB* transcription derived by the promoter within *crtI*. Therefore, two strategies were applied to create phytoene-accumulating strains: enzymatic digestion and error-prone PCR. Both methodologies were applied to knock out the gene *crtI* on pAC-LYC and to dysfunction phytoene desaturase, resulting in two plasmids, pAC-PTE-TR (representing "truncated") and pAC-PTE-MT (representing "mutated"), respectively (Figure 4A). A total of 948 base pairs of *crtI* open reading frame (ORF) (65% of *crtI*) were removed from pAC-LYC to create pAC-PTE-TR, whereas the pAC-PTE-MT maintained the same length of ORF as pAC-LYC but had mutations in *crtI*. The mutated *crtI* ORF in the pAC-PTE-MT was sequenced, and the result showed that 101 amino acids were deleted from the protein and 3 amino acids were inserted into the protein by mutations (Figure 4B).

Both plasmids were introduced into BL21Star(DE3), and the phytoene accumulation patterns were evaluated. Both plasmids resulted in phytoene accumulation, and both strains, which were cultured in LB broth, accumulated higher concentrations of carotenoids than in minimal medium. Interestingly, *E. coli* harboring pAC-PTE-MT accumulated more phytoene than the one harboring pAC-PTE-TR (Figure 5). The transcription of either GGDP synthase (*crtE*) or phytoene synthase (*crtB*) was potentially affected by the enzyme digestion of *crtI*, and therefore real-time PCR was used to evaluate the total RNA level of *crtE* or *crtB* among tested strains. The RNA expression of *crtE* was slightly higher in pAC-PTE-TR compared with pAC-LYC or pAC-PTE-MT (Figure 6A), but the expression of *crtB* was significantly decreased (Figure 6B).

In this study, the deletion of *crtI* ORF reduced the mRNA transcription of *crtB* and further resulted in decreased phytoene accumulation, suggesting that the promoter within the *crtI* ORF is involved in the transcription of *crtB*. Using error-prone PCR methodology, a mutated *crtI* that potentially maintained the promoter region within *crtI* was generated; this promoter maintained the transcription of *crtB* and resulted in a higher phytoene accumulation compared with truncated *crtI*. In addition, there may be a polar effect between *crtI* and *crtB* in which the mRNA is more stable if the two genes are transcribed together. Therefore, error-prone PCR rescued phytoene production by maintaining *crtB* transcription, and the *E. coli* harboring pAC-PTE-MT was selected as the model for further ^{13}C-labeling.

In addition, hundreds of mutant colonies harboring mutated *crtI* as well as functional *crtB* and *crtE* were screened by UV lamp to locate a phytofluene-accumulating colony, but none of the colonies was identified to accumulate phytofluene. All screened mutants accumulated phytoene. Many studies have characterized diverse products by combining *crtI*-type phytoene desaturases from different species in *E. coli*.[29] Although the active site of phytoene desaturase from *E. agglomerans* has not been thoroughly studied, on the basis of our error-prone PCR results, we suspect that once the active site is disabled, the enzyme entirely loses its function and is unable to catalyze any desaturation reaction. Error-prone PCR could not generate a mutated phytoene desaturase that catalyzed only one, two, or three steps of the desaturation cascade.

Phytoene Accumulation in BL21Star(DE3). *E. coli* strain BL21Star(DE3) harboring pAC-PTE-MT was grown in 2XM9 minimal medium containing 5 g/L ^{12}C-glucose, and its growth characteristics were similar to those of the strain harboring pAC-LYC (Figure 7A). Over 60% of glucose was used between 12 and 24 h, and the wet cell weight reached its maximum at 24 h. The total phytoene accumulation per 5 mL of culture reached 836 ng by 24 h and further reached 896 ng by 36 h (Figure 7B). Accumulated phytoenes were mainly 15-*cis*- and *all-trans*-phytoene, and no other phytoene isomers were detected. Interestingly, the phytoene accumulation per gram of wet cells reached its steady state at 12 h and maintained a similar level, indicating that phytoene accumulation was taking place while *E. coli* was growing. After *E. coli* reached its maximum cell mass, the synthesis of phytoene stopped, which is different from the pattern observed with lycopene accumulation. A trace amount of acetate was also accumulated in the media at 24 h and depleted by 48 h.

Phytoene synthase catalyzes a condensation reaction and yields phytoene, whereas phytoene desaturase catalyzes desaturation reactions and yields lycopene. In general, a desaturation reaction is not spontaneous but requires an energizing component. Unlike fatty acid desaturation, which yields an isolated double bond by NADH-dependent hydroxylation and water abstraction, desaturation of phytoene and other carotenes is thermodynamically favored due to the resulting extension of the conjugated double bond system, which is an exergonic reaction.[29] Therefore, the desaturation reaction might be continued even though the glucose was depleted in the media, whereas the condensation reaction was stopped.

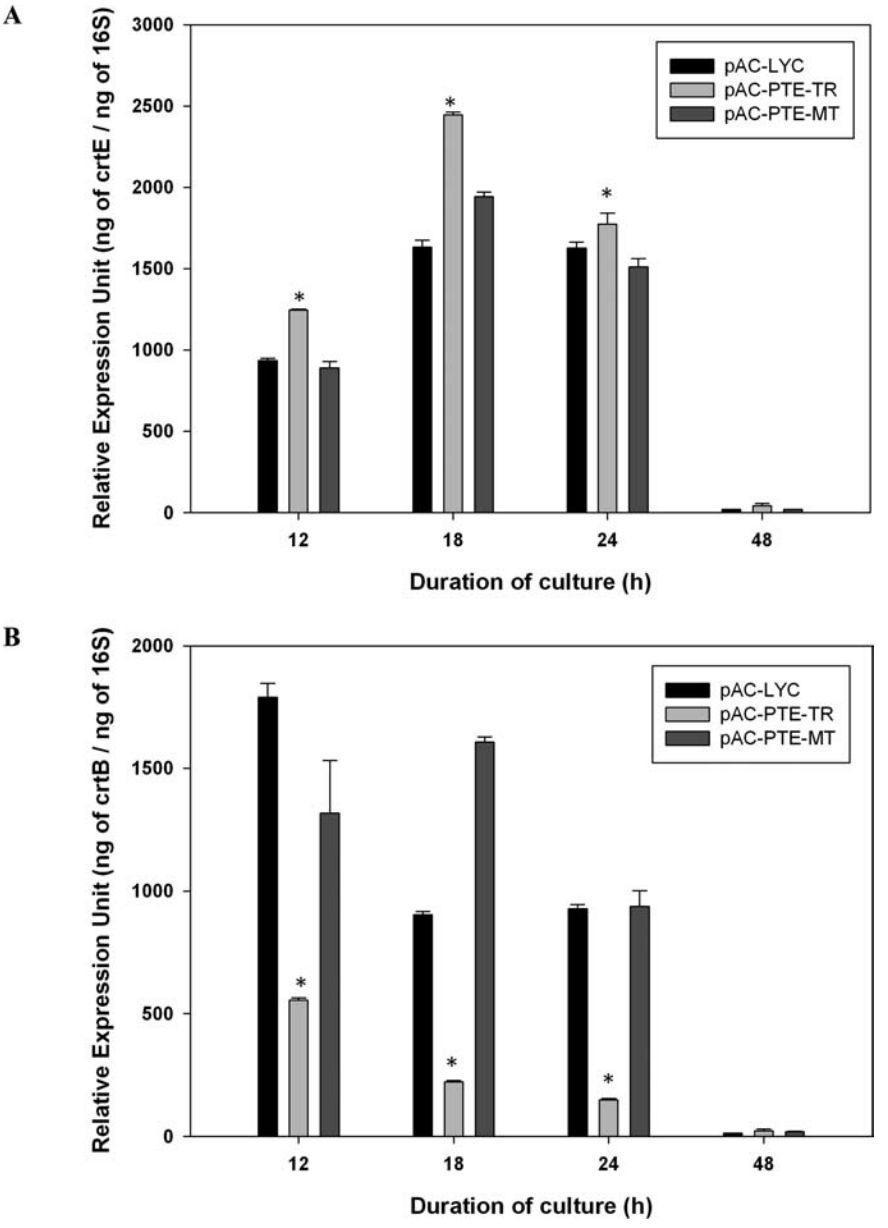

Figure 6. RNA expression patterns of *E. coli* harboring pAC-LYC, pAC-PTE-TR, and pAC-PTE-MT in LB broth: (A) GGPP synthase (*crtE*); (B) phytoene synthase (*crtB*). Data are presented as the mean ± SE with $n = 3$. ∗, $p < 0.05$.

Isotopomer Distribution of ^{13}C-Labeled Phytoene. Mass spectrometry showed that the ion m/z 544.3 was the major ion for the unlabeled phytoene, followed by m/z 545.2 and 546.3 (Figure 8), which was similar to what we observed with unlabeled lycopene sample. Comparing mass spectra of labeled phytoene with unlabeled phytoene, we observed the majority of ions shifted to m/z 584.4 [M + 40$^{\bullet}$], followed by the ion m/z 583.4 [M + 39$^{\bullet}$], which indicates that the biosynthesized phytoene is enriched with ^{13}C. Mass spectrometry of phytoene was performed using APCI under positive mode, which led to a detectable signal, whereas ESI did not. A similar finding was reported by Rivera et al.[27]

Advantages and Disadvantages of Using *E. coli* To Produce Isotopically Labeled Carotenoids. Bioengineered *E. coli* has the potential to generate a wide variety of carotenoids by combining different carotenogenic genes from different bacteria. For example, combining phytoene synthase from *E. agglomerans* and phytoene desaturase from a *Rhodobacter* species that catalyzes only three steps of desaturation would generate molecules such as β-zeacarotene and hydroxyneurosporene, instead of lycopene.[30] Furthermore, molecular breeding or in vitro evolution techniques have also been applied to create novel carotenoids in *E. coli*. Schmidt-Dannert et al. expressed a shuffled phytoene desaturase in *E. coli* and created a fully conjugated carotenoid, 3,4,3′,4′-tetradehydrolycopene.[19] The C30 pathway was also tested, and a novel cyclic carotenoid, C_{30}-diapotorulene, was produced.[31] Therefore, by utilizing an *E. coli* system, labeled carotenoids with novel or rare structures may be easily biosynthesized. In addition, because [U]-^{13}C-glucose is the only carbon source in the minimal medium, ^{12}C carry-over could be low, resulting in highly uniformly labeled products.

E. coli is relatively easy to maintain and culture for a biosafety level-2 laboratory, and the operation can be managed by an

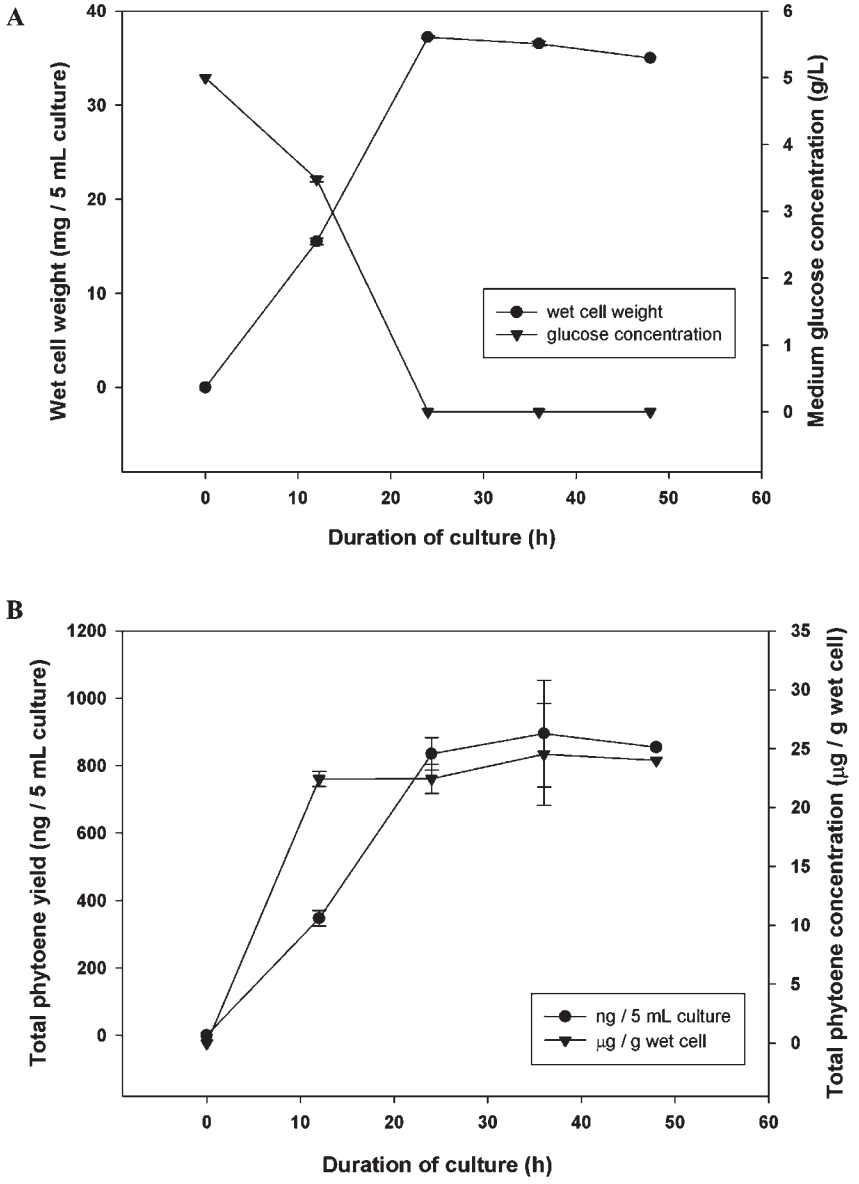

Figure 7. Characteristics of *E. coli* BL21Star(DE3) harboring pAC-PTE-MT: (A) cell growth and corresponding glucose concentration of 2XM9 media; (B) accumulation of total phytoene. Data are presented as the mean ± SE with $n = 3$.

entry-level scientist. In addition, laboratory-scale production can be performed on site, which can provide freshly biosynthesized carotenoids to avoid oxidized or degraded contaminants. In contrast, chemical synthesis of carotenoids is lengthy, and it requires experienced organic chemists and a sophisticated organic chemistry laboratory. For mass spectrometry internal standard purposes, *E. coli* produces customized labeled carotenoids in 36 h, and the purification process is relatively simple compared with the extractions from other biotechnological production systems, such as tomato cell culture[32] or microalgae.[33]

However, like every biotechnological production system, byproducts can be produced and extracted during the process, requiring further purification with liquid chromatography. Also, unlike chemical synthesis, it is challenging to label specific atoms in the biosynthesized molecules, because special labeled precursors could be costly. For this model system, one 5 mL culture produced 1.4 μg of ^{13}C-labeled lycopene, which costs an estimated $1.25. Although this model system may not be cost-effective, research has been devoted to improve carotenoid production. For example, the supply of carotenoid synthesis precursor IPP had been improved by Kim et al.,[34] which doubled the lycopene production. Introducing the mevalonate pathway resulted in a 10-fold increase in lycopene production.[35] Gene knockout strategies have also been applied to improve lycopene production, resulting in a strain that can accumulate 16 mg of lycopene/g dry cell mass.[23−25] These metabolic-engineered strains could potentially make the *E. coli* system cost-effective for labeled carotenoid production.

Summary. This research demonstrated that bioengineered *E. coli* could utilize [U]-^{13}C-glucose to produce highly ^{13}C-enriched tomato carotenoids, lycopene and phytoene, and that error-prone PCR maintained phytoene production by maintaining the transcription of phytoene synthase. This production system could be further optimized and provide sufficient labeled

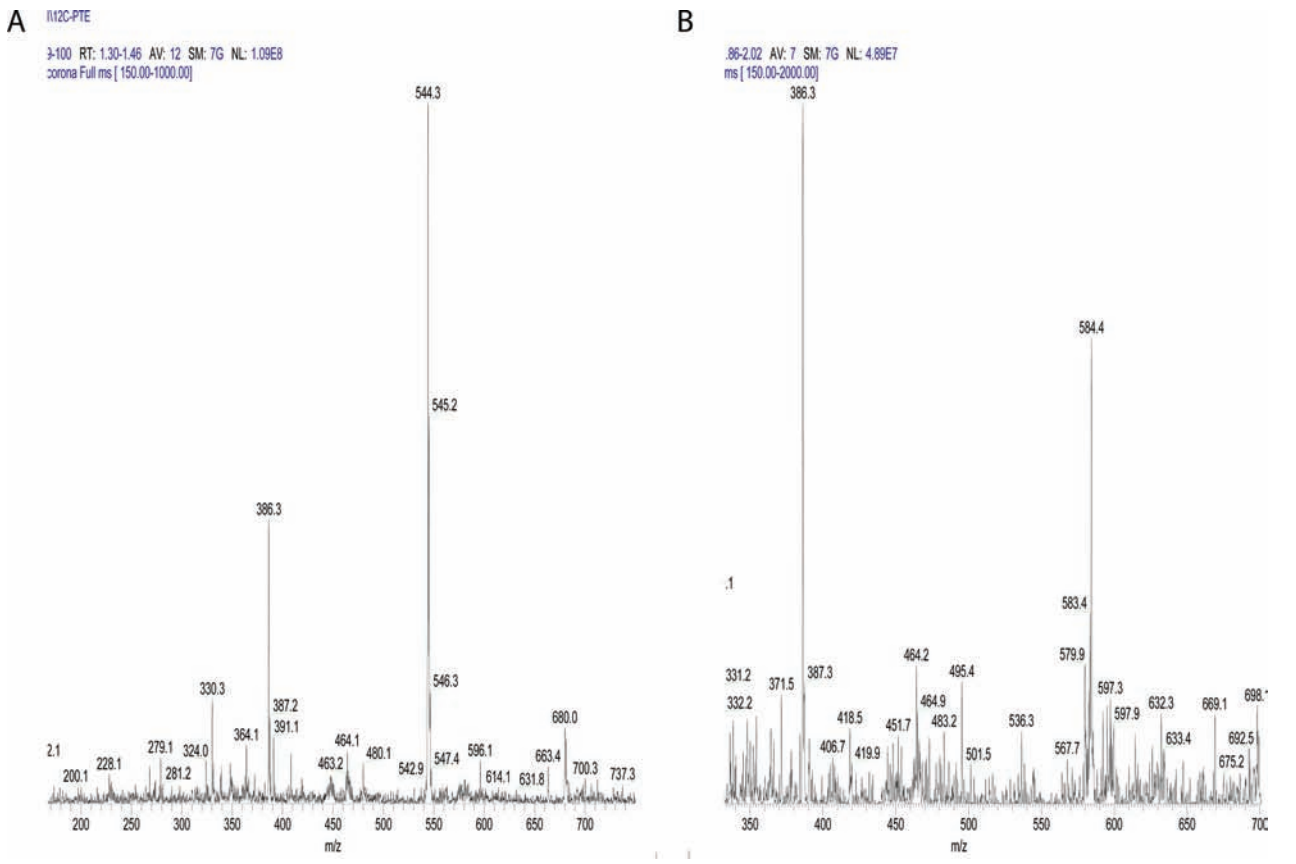

Figure 8. Isotopomer distribution of (A) ^{12}C-phytoene ($^{12}C_{40}H_{64}$, MW = 544.3) and (B) ^{13}C-labeled phytoene ($^{13}C_{40}H_{64}$, MW = 584.4), measured by ThermoFinnigan LCQ Deca XP with APCI+ interface.

carotenoids for future absorption, distribution, metabolism, and excretion studies of carotenoids.

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REFERENCES

(1) Canene-Adams, K.; Campbell, J. K.; Zaripheh, S.; Jeffery, E. H.; Erdman, J. W., Jr. The tomato as a functional food. *J. Nutr.* **2005**, *135*, 1226–1230.

(2) Breemen, R. B.; van Pajkovic, N. Multitargeted therapy of cancer by lycopene. *Cancer Lett.* **2008**, *269*, 339–351.

(3) Parker, R. S.; Swanson, J. E.; Marmor, B.; Goodman, K. J.; Spielman, A. B.; Brenna, J. T.; Viereck, S. M.; Canfield, W. K. Study of β-carotene metabolism in humans using ^{13}C-β-carotene and high precision isotope ratio mass spectrometry. *Ann. N.Y. Acad. Sci.* **1993**, *691*, 86–95.

(4) Yao, L.; Liang, Y.; Trahanovsky, W. S.; Serfass, R. E.; White, W. S. Use of a ^{13}C tracer to quantify the plasma appearance of a physiological dose of lutein in humans. *Lipids* **2000**, *35*, 339–348.

(5) Novotny, J. A.; Dueker, S. R.; Zech, L. A.; Clifford, A. J. Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. *J. Lipid Res.* **1995**, *36*, 1825–1838.

(6) Tang, G.; Qin, J.; Dolnikowski, G. G.; Russell, R. M. Vitamin A equivalence of β-carotene in a woman as determined by a stable isotope reference method. *Eur. J. Nutr.* **2000**, *39*, 7–11.

(7) Verdegem, P. J.; Bovee-Geurts, P. H.; de Grip, W. J.; Lugtenburg, J.; de Groot, H. J. Retinylidene ligand structure in bovine rhodopsin, metarhodopsin-I, and 10-methylrhodopsin from internuclear distance measurements using ^{13}C-labeling and 1-D rotational resonance MAS NMR. *Biochemistry* **1999**, *38*, 11316–24.

(8) Lienau, A.; Glaser, T.; Tang, G.; Dolnikowski, G. G.; Grusak, M. A.; Albert, K. Bioavailability of lutein in humans from intrinsically labeled vegetables determined by LC-APCI-MS. *J. Nutr. Biochem.* **2003**, *14*, 663–670.

(9) Borowitzka, M. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *J. Biotechnol.* **1999**, *70*, 313–321.

(10) Carvalho, A. P.; Meireles, L. A.; Malcata, F. X. Microalgal reactors: a review of enclosed system designs and performances. *Biotechnol. Prog.* **2006**, *22*, 1490–1506.

(11) Campo, J. A.; García-González, M.; Guerrero, M. G. Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. *Appl. Microbiol. Biottechnol.* **2007**, *74*, 1163–1174.

(12) Campbell, J. K.; Rogers, R. B.; Lila, M. A.; Erdman, J. W., Jr. Biosynthesis of ^{14}C-phytoene from tomato cell suspension cultures (*Lycopersicon esculentum*) for utilization in prostate cancer cell culture studies. *J. Agric. Food Chem.* **2006**, *54*, 747–755.

(13) Engelmann, N. J.; Rogers, R. B.; Lila, M. A.; Erdman, J. W., Jr. Herbicide treatments alter carotenoid profiles for ^{14}C tracer production from tomato (*Solanum lycopersicum* cv. VFNT cherry) cell cultures *J. Agric. Food Chem.* **2009**, *57*, 4614–4619.

(14) Engelmann, N. J.; Campbell, J. K.; Rogers, R. B.; Rupassara, S. I.; Garlick, P. J.; Lila, M. A.; Erdman, J. W., Jr. Screening and selection of high carotenoid producing in vitro tomato cell culture lines for [^{13}C]-carotenoid production. *J. Agric. Food Chem.* **2010**, *58*, 9979–9987.

(15) Perry, K. L.; Simonitch, T. A.; Harrison-Lavoie, K. J.; Liu, S. T. Cloning and regulation of *Erwinia herbicola* pigment genes. *J. Bacteriol.* **1986**, *168*, 607–612.

(16) Armstrong, G. A.; Alberti, M.; Hearst, J. E. Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9975–9979.

(17) Harada, H.; Misawa, N. Novel approaches and achievements in biosynthesis of functional isoprenoids in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 1021–1031.

(18) Sandmann, G. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *Chembiochem* **2002**, *3*, 629–635.

(19) Schmidt-Dannert, C.; Umeno, D.; Arnold, F. H. Molecular breeding of carotenoid biosynthetic pathways. *Nat. Biotechnol.* **2000**, *18*, 750–753.

(20) Umeno, D.; Tobias, A. V.; Arnold, F. H. Diversifying carotenoid biosynthetic pathways by directed evolution. *Microbiol. Mol. Biol. Rev.* **2005**, *69*, 51–78.

(21) Yoon, S.-H.; Lee, S.-H.; Das, A.; Ryu, H.-K.; Jang, H.-J.; Kim, J.-Y.; Oh, D.-K.; Keasling, J. D.; Kim, S.-W. Combinatorial expression of bacterial whole mevalonate pathway for the production of β-carotene in *E. coli*. *J. Biotechnol.* **2009**, *140*, 218–226.

(22) Albrecht, M.; Misawa, N.; Sandmann, G. Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. *Biotechnol. Lett.* **1999**, *21*, 791–795.

(23) Alper, H.; Jin, Y.-S.; Moxley, J. F.; Stephanopoulos, G. Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* **2005**, *7*, 155–164.

(24) Jin, Y.-S.; Stephanopoulos, G. Multi-dimensional gene target search for improving lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* **2007**, *9*, 337–47.

(25) Alper, H.; Stephanopoulos, G. Uncovering the gene knockout landscape for improved lycopene production in *E. coli*. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 801–810.

(26) Cunningham, F. X.; Sun, Z.; Chamovitz, D.; Hirschberg, J.; Gantt, E. Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942. *Plant Cell* **1994**, *6*, 1107–1121.

(27) Rivera, S.; Vilaró, F.; Canela, R. Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants. *Anal. Bioanal. Chem.* **2011**, *400*, 1339–1346.

(28) To, K. Y.; Lai, E. M.; Lee, L. Y.; Lin, T. P.; Hung, C. H.; Chen, C. L.; Chang, Y. S.; Liu, S. T. Analysis of the gene cluster encoding carotenoid biosynthesis in *Erwinia herbicola* Eho13. *Microbiology* **1994**, *140*, 331–339.

(29) Sandmann, G. Evolution of carotene desaturation: the complication of a simple pathway. *Arch. Biochem. Biophys.* **2009**, *483*, 169–174.

(30) Sandmann, G.; Albrecht, M.; Schnurr, G.; Knörzer, O.; Böger, P. The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*. *Trends Biotechnol.* **1999**, *17*, 233–237.

(31) Lee, P. C.; Momen, A. Z. R.; Mijts, B. N.; Schmidt-Dannert, C. Biosynthesis of structurally novel carotenoids in *Escherichia coli*. *Chem. Biol.* **2003**, *10*, 453–462.

(32) Lu, C.-H.; Engelmann, N. J.; Lila, M. A.; Erdman, J. W., Jr. Optimization of lycopene extraction from tomato cell suspension culture by response surface methodology. *J. Agric. Food Chem.* **2008**, *56*, 7710–7714.

(33) Wilson, P. D. G.; Hilton, M. G.; Waspe, C. R.; Steer, D. C.; Wilson, D. R. Production of C-13-labelled β-carotene from *Dunaliella salina*. *Biotechnol. Lett.* **1997**, *19*, 401–405.

(34) Kim, S. W.; Keasling, J. D. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng.* **2001**, *72*, 408–415.

(35) Rodríguez-Villalón, A.; Pérez-Gil, J.; Rodríguez-Concepción, M. Carotenoid accumulation in bacteria with enhanced supply of isoprenoid precursors by upregulation of exogenous or endogenous pathways. *J. Biotechnol.* **2008**, *135*, 78–84.