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Improvement of a CrtO-type of β-carotene ketolase for canthaxanthin production in *Methylomonas* sp.

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Abstract

Two types of non-homologous β-carotene ketolases (CrtW and CrtO) were previously described. We report improvement of a CrtO-type of β-carotene ketolase for canthaxanthin production in a methylotrophic bacterium, *Methylomonas* sp. 16a, which could use the C1 substrate (methane or methanol) as sole carbon and energy source. The *crtO* gene from *Rhodococcus erythropolis* was improved for canthaxanthin production in an *E. coli* strain engineered to produce high titer carotenoids by error-prone PCR mutagenesis followed by in vitro recombination. The best mutants from protein engineering could produce ~90% of total carotenoids as canthaxanthin in the high titer *E. coli* strain compared to ~20% canthaxanthin produced by the starting gene. Canthaxanthin production in *Methylomonas* was also significantly improved to ~50% of total carotenoids by the mutant genes. Further improvement of canthaxanthin production to ~93% in *Methylomonas* was achieved by increased expression of the best mutant gene. Some mutations were found in many of the improved genes, suggesting that these sites, and possibly the regions around these sites, were important for improving the *crtO*'s activity for canthaxanthin production.

Keywords: Carotenoid ketolase; Ketocarotenoids; Methanotroph; Protein engineering; In vitro recombination

1. Introduction

Canthaxanthin (β,β′-caroten-4,4′-dione) is one of the high value ketocarotenoids that has been widely used as a feed supplement in poultry and aquaculture industries for pigmentation. Most of the industrially used canthaxanthin is synthesized chemically, although some are derived from biological sources such as *Dietzia maris* (formerly *Rhodococcus maris*) (Shahidi et al., 1998). Canthaxanthin produced from recombinant bacteria provides another competitive source for these applications. Obligate methanotrophs utilize methane or methanol as sole carbon and energy source. They have been exploited for commercial production of single cell proteins (Tuse, 1984). Production of high value compounds such as canthaxanthin or astaxanthin (3,3′-dihydroxy-β,β′-caroten-4,4′-dione) from the inexpensive C1 feedstocks attracted our interests.

Canthaxanthin is synthesized by addition of two keto groups to β-carotene (β,β′-carotene) precursor. The β-carotene precursor can be generated from farnesyl pyrophosphate by sequential reactions catalyzed by four enzymes encoded by *crtEBIY* genes (Fig. 1). Carotenoid synthesis gene clusters containing *crtEBIY* genes were reported in *Enterobacteriaceae* strains, several of which contained an additional isoprenoid gene *idi* encoding isopentenyl pyrophosphate isomerase in the cluster (Sedkova et al., 2005). The β-carotene ketolases responsible for keto group addition to the β-ionone rings were found in some bacteria (Misawa et al., 1995; Fernandez-Gonzalez et al., 1997; Hannibal et al., 2000; Steiger and Sandmann, 2004; Tao and Cheng, 2004; Choi et al., 2005) and algae (Kajiwara et al., 1995). Most of the β-carotene ketolases are CrtW-type enzymes, which were frequently used for efficient production of canthaxanthin and astaxanthin in heterologous hosts (Steiger and Sandmann, 2004; Choi et al., 2005). Recently, a new type of β-carotene ketolase (CrtO) was reported in cyanobacteria (Fernandez-Gonzalez et al., 1997; Mochimaru et al., 2005) and

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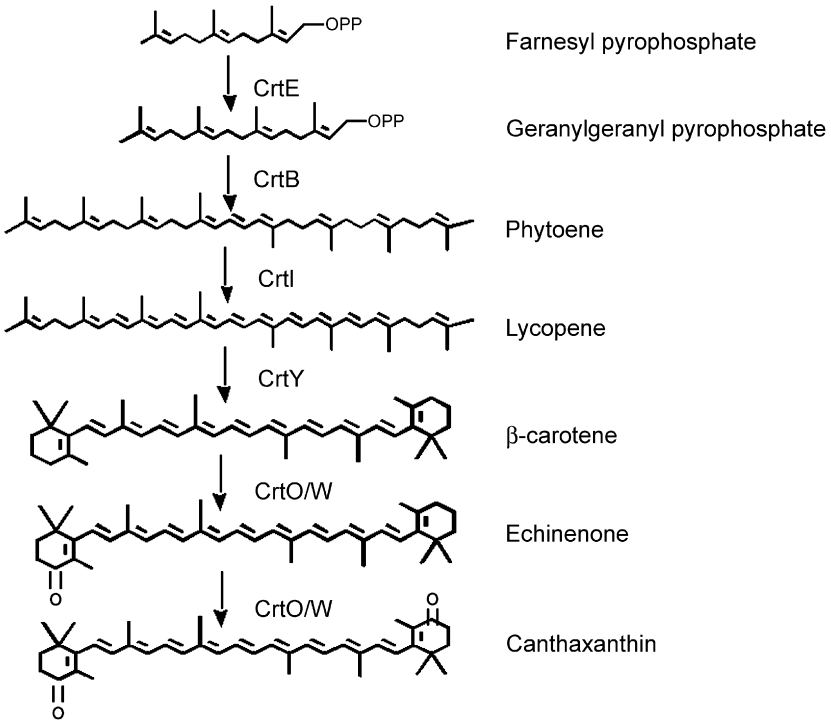

Fig. 1. Canthaxanthin synthesis pathway. CrtE: geranylgeranyl pyrophosphate synthase; CrtB: phytoene synthase; CrtI: phytoene dehydrogenase; CrtY: lycopene β-cyclase; CrtO: β-carotene ketolase; CrtW: β-carotene ketolase.

non-photosynthetic bacteria (Tao and Cheng, 2004). The CrtO enzymes (>500 amino acids) are almost twice the size as the CrtW enzymes and share no sequence homology with the CrtW enzymes.

In this article, we report the combined use of protein engineering and increased gene expression to improve a *crtO* from *Rhodococcus erythropolis* for canthaxanthin production in a methanotrophic bacterium *Methylomonas* sp.

2. Materials and methods

2.1. Growth conditions

Derivatives of DH10B, WS162 and WS210 (Yuan et al., 2006) *Escherichia coli* cells were routinely grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) with appropriate antibiotics at 37 °C shaking overnight. For carotenoids analysis, they were grown at 30 °C shaking for 2–3 days. *Methylomonas* sp. 16a (ATCC PTA-2402) derivatives were grown in 10 ml BTZ mineral medium (Odom et al., 1991) with 50 μg/ml kanamycin in 160 ml-serum bottles (Wheaton Scientific, Wheaton IL). The bottles were filled with 25% methane in air as a sole carbon and energy source and closed by gas-tight butyl septa. The *Methylomas* cultures were growing by shaking at 250 rpm for 2–3 days at 30 °C.

2.2. Construction of broad host range canthaxanthin-producing plasmids

Pantoea stewartii contains a natural carotenoid synthesis gene cluster *crtEXYIBZ* (accession number AY166713). The genes required for β-carotene synthesis (i.e., *crtE* and *crtYIB*) were joined together by overlapping PCR. A unique MfeI site was engineered in the intergenic region of *crtE* and *crtY*. The ~4.5 kb EcoRI fragment containing the joined *crtEYIB* gene cluster was ligated into a unique EcoRI site of a broad host range vector pBHR1 (MoBiTec GmbH, Goettingen, Germany), to create the β-carotene-synthesis plasmid pDCQ301. Three canthaxanthin-synthesis plasmids pDCQ303, pDCQ319, and pDCQ320 were prepared by cloning variants of a *crtO* gene from *Rhodococcus erythropolis* (accession number AY705709) into the unique MfeI site of pDCQ301 downsteam of the *crtE* gene. Plasmid pDCQ319 contained a synthetic *crtO* gene which was codon optimized for expression in *Methylomonas*. The *crtO319* gene was synthesized by Aptagen (Herndon, VA). Plasmid pDCQ320 contained a modified *crtO* gene, which optimized only several codons at the 5′ and 3′ ends of the gene by PCR primers. Plasmid pDCQ303 contained the same *crtO* coding sequence as *crtO320* except that three additional amino acids (Met-Ala-Leu) were fortuitously introduced at the N-terminus by the PCR primer. The three plasmids pDCQ303, pDCQ319 and pDCQ320 all contained the *crtEOYIB* gene cluster expressed under the *Pcat* promoter in pBHR1 vector. One control plasmid pDCQ307 was prepared by cloning a *crtW* from *Paracoccus* sp. N81106 (accession number D58420) into pDCQ301 downstream of the *crtE* gene.

Another canthaxanthin-producing plasmid was constructed using a different β-carotene synthesis gene cluster *crtEidiYIB* from *Pantoea agglomerans* DC404 (accession number DQ090834). An improved *crtO* gene (crtO* =

320SHU001) was cloned upstream of the *crtE* gene, creating the plasmid pDCQ354 expressing the *crtO*EidiYIB* gene cluster under the *Pcat* promoter in pBHR1 vector.

2.3. Error-prone PCR library construction

The various *crtO* genes (*crtO*303, *crtO*319, and *crtO*320) were removed from the constructs using MfeI and XbaI double digestion. Three random mutant libraries targeting the entire *crtO* gene were made for all three constructs. Error-prone PCR was performed using a Diversify PCR mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, CA) following procedure in condition 4. The PCR primers used were:

for pDCQ319:

crtO-For (5′-AGCCAATTGAAGGAGGAATAAACCATG-3′)
crtO-Rev (5′-GCGAATTCCTCTAGATTAGCTACGGCT-3′)

for pDCQ303:
crtO900-For 5′-TAACAATTGAAGGAGGAATAAACCATGGCC-3′
crtO303-Rev 5′-GCGAATTCCTCTAGATCACGAGCGGCTCGA-3′

for pDCQ320:
320-F1 5′-GCCATTAGCCAGACCGGCA-3′
320-R1 5′-GCGCCTGGCCAGTGAACA-3′

The 1.6 kb error-prone PCR products were digested with MfeI and XbaI, and ligated with the MfeI/XbaI-digested vectors that removed the *crtO* insert. The ligation mixture was first transformed into XL1-Blue MRF' *E. coli* cells (Stratagene, La Jolla, CA) by electroporation. After growing the mutant library cells in LB liquid media or on LB agar plates containing kanamycin, the plasmids were pooled from the library cells and retransformed into the high titer *E. coli* WS210 cells for screening.

2.4. In vitro recombination

The hits obtained from the error-prone PCR mutagenesis screening of CrtO320 library together with the CrtO320 itself were used as parents for in vitro recombination (Ikeuchi et al., 2003; Milano and Tang, 2004) to generate more variants (Fig. 2). The 5′ ends of one set of parents (gene set 1) were extended by standard high fidelity PCR using primers 320SUUP-F1: 5′-TTT CAA GCT ATA CCA AGC ATA CAA TGA CAA TTG AAG GAG GAA TAA ACC ATG-3′ and 320SUUP-R1: 5′-GCG AAT TCC TGT AGA TCA CGA GCG GCT CGA-3′. The 3′ ends of another set of parents (gene set 2) were similarly extended using primers 320SUUP-F2: 5′-AGC GAA TTG AAG GAG GAA TAA ACC ATG-3′ and 320SUUP-R2: 5′-GAG GCT AGT TAT TGC TCA GCG GTC TAG ATC ACG AGC GGC TCG A-3′. The purified two pools of PCR products were used as templates for generation of recombinant DNA products in vitro. Two unpaired primers pADH316-F1: 5′-CAA GCT ATA CCA AGC

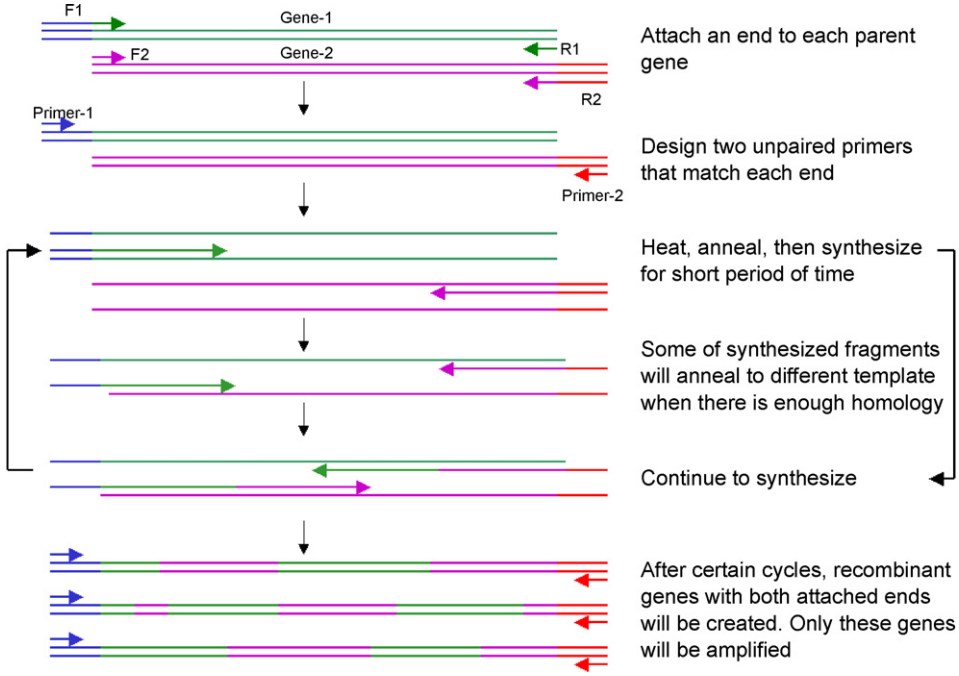

Fig. 2. Schematic illustration of in vitro recombination. The gene variants were shown as double-stranded green lines and double-stranded purple lines. The 5′ extended region shown as double-stranded blue lines was attached by PCR using 320SUUP-F1 and 320SUUP-R1. The 3′ extended region shown as double-stranded red lines was attached by PCR using 320SUUP-F2 and 320SUUP-R2. The in vitro recombinogenic PCR was performed using the two unpaired primers 1 and 2.

ATA CAA TGA-3′ and T7T: 5′-GCT AGT TAT TGC TCA GCG G-3′ were used for recombinogenic PCR. Primer pADH316-F1 annealed to the 5′ extended region of set 1 parents, but not to the 5′ template of set 2 parents. Likely, primer T7T annealed to the 3′ extended region of set 2 parents, but not to the 3′ template of set 1 parents. Thermocycling condition was: 95 °C denaturation for 10 min; followed by 60 cycles of 1 min at 94 °C, 5 s at 63–69 °C temperature gradient across 12 wells with increment of 1 s of each cycle; final extension at 72 °C for 7 min; and hold at 4 °C. The reaction mixture was gel purified and cloned into pDCQ320 replacing the *crtO* gene. The ligation mixture was first transformed into XL1-Blue MRF' *E. coli* cells by electroporation. After growing the mutant library cells in LB containing kanamycin at 37 °C for 12 h, the plasmids were pooled from the library cells and retransformed into the high titer *E. coli* WS210 cells for screening.

2.5. Triparental conjugation

Triparental conjugation was performed to transfer plasmids from *E. coli* donors to derivatives of *Methylmonas* sp. strain 16a. Overnight cultures of the *E. coli* donor strain containing the plasmid of interest, the *E. coli* helper strain containing pRK2013 (ATCC 37159) and the *Methylomonas* recipient strain were used at a ratio of 1:1:5 based on volume. The cells grown overnight in the presence of appropriate antibiotics were collected by centrifugation and washed with BTZ medium. The cells of donor, helper, and recipient strains were combined and concentrated in 50 μl of BTZ medium. The mixture was spotted onto a BTZ agar plate supplemented with 0.5% yeast extract. The plate was incubated for three days at 30 °C in an AnaeroPack™ box filled with 25% methane. The cells grown on the plate were then resuspended and spread onto BTZ agar plates supplemented with kanamycin (50 μg/ml). Transconjugants were obtained after incubation the plates for about 5 days. They were purified by repeated streak onto fresh BTZ plates with kanamycin.

2.6. Carotenoid analysis

HPLC and LC/MS analysis of carotenoids from *E. coli* and *Methylomonas* cells were performed as described previously (Tao et al., 2005). The β-carotene standard was purchased from Sigma (St. Louis, MO), and the canthaxanthin standard was purchased from CaroteNature (Lupsingen, Switzerland).

2.7. Nucleotide accession number

The nucleotide sequences of the modified *crtO* genes were deposited into GenBank under accession numbers EF534197-EF534199.

3. Results and discussion

3.1. Selection of the starting crtO gene and the screening host for protein engineering

The recently identified *crtO* genes encode a new family of β-carotene ketolases that are non-homologous to the *crtW*-encoded β-carotene ketolases (Fernandez-Gonzalez et al., 1997; Tao and Cheng, 2004). We were interested in producing canthaxanthin using a CrtO-type of β-carotene ketolase. The *crtO* genes from *Synechocystis* sp. (slr0088), *Rhodococcus erythropolis* (AY705709) and *Deinococcus radiodurans* (DR0093) were compared. The *Rhodococcus erythropolis crtO* was chosen since it showed the highest activity in *E. coli* (Tao and Cheng, 2004). The coding sequence of the wild-type *Rhodococcus crtO* was 1599 bp with a GTG as the putative start codon. The high GC content of the *crtO* gene from *Rhodococcus* was modified to facilitate expression in gram-negative bacteria. Three canthaxanthin-producing plasmids, pDCQ319, pDCQ303 and pDCQ320, were created containing different variants of the *Rhodococcus crtO* gene with ATG as the start codon (see Section 2). Error-prone PCR mutagenesis libraries were constructed using the three *Rhodococcus crtO* variants (see Section 2).

The obligate methylotrophic bacterium *Methylomonas* sp. 16a (ATCC PTA-2402) was our choice of production host for canthaxanthin, since it grew on inexpensive C1 feedstocks such as methane or methanol. However, the limited genetic tools for manipulation of *Methylomonas* prevented us from using it directly as the screening host for protein engineering. To search for a surrogate host for screening, we compared canthaxanthin production in *Methylomonas* and different *E. coli* strains. A mutant derivative of *Methylomonas* sp. 16a that did not produce its native C_{30} pigment (Sharpe et al., 2007) was used in this study. This *Methylomonas* strain MWM1200 (ATCC PTA-6887) could produce about 1200 ppm of total C_{40} carotenoids when the exogeneous C_{40} synthesis genes were introduced. Three *E. coli* strains, DH10B, WS162 and WS210 (Yuan et al., 2006), were included for comparison. DH10B was an unengineered *E. coli* that was capable of producing about 200 ppm of total C_{40} carotenoids. WS162 was an engineered strain with increased expression of several isoprenoid synthesis genes (PT5-*dxs*, PT5-*idi*, PT5-*ispDF*) and was capable of producing about 3000 ppm of total C_{40} carotenoids. WS210 was another engineered strain with increased expression of additional isoprenoid synthesis genes (PT5-*dxs*, PT5-*idi*, PT5-*ispDF*, PT5-*ispB*) and was capable of producing about 6000 ppm of total C_{40} carotenoids. Table 1 showed the percentage of canthaxanthin and echinenone converted from β-carotene by the CrtO on pDCQ303 and by the CrtW on pDCQ307 in different hosts. The CrtW ketolase showed high activity in the *E. coli* and *Methylomonas* hosts suggesting that no inhibiting agent or limiting factor was present for the ketolase activity in all the hosts. In the low titer *E. coli*

Table 1
Conversion of β-carotene by the CrtW and CrtO ketolases in different hosts

Strain[b]	CrtW[a]		CrtO[a]	
	CTX%[c]	ECH%[c]	CTX%	ECH%
E. coli				
DH10B	100	0	84	11
WS162	96	2	44	34
WS210	92	5	12	18
Methylomonas				
MWM1200	86	5	14	18

[a]The CrtW on pDCQ307 was from *Paracoccus* sp. N81106 (accession number D58420). The CrtO on pDCQ303 was from *Rhodococcus erythropolis* (accession number AY705709).

[b]The *E. coli* strains, DH10B, WS162 and WS210, were capable of producing about 200, 3000 and 6000 ppm of C_{40} carotenoids, respectively. The *Methylomonas* strain MWM1200 was capable of producing about 1200 ppm of C_{40} carotenoids.

[c]Percentage yield of canthaxanthin (CTX) and echinenone (ECH) in the total carotenoids. The remaining carotenoid in the cells was β-carotene.

strain, the CrtO303 could convert more than 80% of β-carotene to canthaxanthin. However, less amount of canthaxanthin was produced in the high titer *E. coli* strains indicating insufficient ketolase activity of CrtO for the increased amount of β-carotene substrate. Similar production profile was observed in *Methylomonas* MWM1200 as that of *E. coli* WS210, in which less than 14% of canthaxanthin was produced. *E. coli* strain WS210 was thus chosen as the surrogate screening host for protein engineering of CrtO to improve canthaxanthin production in *Methylomonas*.

3.2. Improvement of canthaxanthin production by protein engineering in E. coli

Protein engineering of the *crtO* gene was achieved by error-prone PCR mutagenesis followed by in vitro recombination of the resulted *crtO* variants. Three error-prone PCR mutagenesis libraries were constructed based on the *crtO* genes in pDCQ303, pDCQ319 and pDCQ320. Ten mutant colonies from each library were randomly picked for sequencing analysis. Many types of base substitutions were present in these mutants, indicating that there was no bias for the mutation type. The mutation rate was 1–5 point mutations per kb of DNA. The frequency of deletion and insertion mutations in the mutant libraries was very low. The enzyme activity distribution was estimated by the color of the mutant colonies. Yellow cells accumulating β-carotene were indicative of inactive ketolase, and orange-red cells producing canthaxanthin were indicative of active ketolase. The results showed that about 40–60% of the mutants in the libraries were active. Approximately 20,000–150,000 mutant colonies from each library were visually screened. The putative hits that showed darker colors than the parental controls were streaked on agar plates. HPLC analysis confirmed that these mutants produced higher percentage of canthaxanthin than the controls (Table 2). The library based on pDCQ320 appeared superior to the libraries based on pDCQ319 and pDCQ303, although improved mutants were isolated from each of the libraries. The majority of the mutants from the *crtO*320 library showed more than 40% of canthaxathin yield. A second round of mutagenesis was performed with the variants isolated from the Crt320 library, using an in vitro recombination technique (Fig. 2) to further improve canthaxanthin yield. The parents for the in vitro recombination were extended with unpaired primers either at the 5′ ends or the 3′ ends. Since the two primers used in the recombination PCR did not match both the 5′ and 3′ ends of either set of templates simultaneously, neither of the parent templates could be amplified during the reaction. However, any recombinant DNA product possessing both the 5′ and 3′ ends of the unpaired regions would be amplified during subsequent thermal cycles. Many mutants (320SHU001–320SHU022) obtained from the second round of in vitro recombination

Table 2
Analysis of canthaxanthin produced from *E. coli* strain WS210 expressing the CrtO genes and the mutants

Strain[a]	Percentage yield of canthaxanthin[b]
CrtO319 (starting gene)	4
319M3022	15
CrtO303(starting gene)	6
303M3044	16
CrtO320(starting gene)	20
320M4019	46
320M4009	46
320M4018	44
320M4007	44
320M4006	43
320M4020	43
320M4036	41
320M4023	40
320M4027	39
320M4032	37
320SHU019	91
320SHU001	89
320SHU017	89
320SHU016	78
320SHU015	68
320SHU008	59
320SHU022	58
320SHU004	56

[a]The top section of the table showed mutants isolated from the three error-prone PCR mutagenesis libraries with CrtO319, CrtO303 or CrtO320 as the starting genes. The bottom section of the table showed mutants isolated from in vitro recombination of the variants obtained from the CrtO320 library.

[b]Percentage yield of canthaxanthin in the total carotenoids. The remaining carotenoids in the cells were β-carotene and echinenone.

Table 3
DNA sequence analysis of mutant *crtO* genes

Strain	Substitutions	Silent mutations
319M3022	GCC(Ala3) to ACC(Thr)	
303M3044	AGC(Ser5) to CGC(Arg)	ACG(Thr498) to ACA(Thr)
320M4006	TCG(Ser449) to ACG(Thr)	GCG(Ala16) to GCA(Ala)
320M4007	TGC(Cys112) to TAC(Tyr) GCG(Ala252) to ACG(Thr) CAG(Gln524) to CGG(Arg)	CTC(Leu17) to CTT(Leu) GCA(Ala24) to GCT(Ala)
320M4009	AGC(Ser2) to AGA(Arg) ACG(Thr37) to ATG(Met)	CGA(Arg117) to CGT(Arg) CCC(Pro444) to CCA(Pro) AGT(Ser505) to AGC(Ser)
320M4018	ACA(Thr147) to TCA(Ser) TCA(Ser464) to ACA(Thr)	CGA(Arg117) to CGT(Arg) GTC(Val133) to GTA(Val) CTG(Leu224) to CTT(Leu) CCC(Pro497) to CCT(Pro)
320M4019	AGC(Ser2) to AAC(Asn) AGT(Ser67) to AAT(Asn) TTT(Phe145) to TCT(Ser) GAC(Asp446) to GGC(Gly)	CGA(Arg302) to CGG(Arg)
320M4020	**ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** ATC(Ile283) to GTC(Val)	
320M4023	AGC(Ser2) to AGA(Arg) GAT(Asp293) to GGT(Gly)	GAC(Asp36) to GAT(Asp)
320M4027	CGG(Arg519) to TGG(Trp)	TCC(Ser331) to TCT(Ser)
320M4031	ACG(Thr304) to AAG(Lys) CAG(Gln524) to CTG(Leu) TCG(Ser529) to TGG(Trp)	GAA(Glu208) to GAG(Glu) CCT(Pro369) to CCC(Pro) GCC(Ala509) to GCT(Ala)
320M4032	GCG(Ala16) to GGG(Gly) TTT(Phe428) to TAT(Tyr)	TTG(Leu240) to CTG(Leu) GAA(Glu276) toGAG(Glu)
320M4036	GCG(Ala252) to ACG(Thr) TCG(Ser264) to CCG(Pro) CAT(His407) to CAA(Gln)	AGC(Ser2) to AGT(Ser) GCA(Ala180) to GCG(Ala)
320SHU001	**ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** ACG(Thr304) to AAG(Lys) CGG(Arg519) to TGG(Trp)	GCG(Ala16) to GCA(Ala) GCA(Ala24) to GCC(Ala) GTG(Leu477) to TTG(Leu)
320SHU004	CGG(Arg519) to TGG(Trp)	GCG(Ala16) to GCA(Ala) TCC(Ser331) to TCT(Ser)
320SHU008	**ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** ATC(Ile283) to GTC(Val)	GCG(Ala16) to GCA(Ala) GCA(Ala24) to GCC(Ala) GTC(Val131) to GTT(Val)
320SHU015	TCG(Ser152) to TTG(Leu) CGG(Arg519) to TGG(Trp)	GCG(Ala16) to GCA(Ala) TCC(Ser331) to TCT(Ser)
320SHU016	ACA(Thr121) to GCA(Ala) **ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** ATC(Ile283) to GTC(Val) CAG(Gln524) to CTG(Leu)	GCG(Ala16) to GCA(Ala) GCA(Ala24) to GCC(Ala) GCC(Ala509) to GCT(Ala)
320SHU017	**ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** ATC(Ile283) to GTC(Val) CGG(Arg519) to TGG(Trp)	GCG(Ala16) to GCA(Ala) GCA(Ala24) to GCC(Ala)
320SHU019	**ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** CGG(Arg339) to CAG(Gln)	GCG(Ala16) to GCA(Ala) GCA(Ala24) to GCC(Ala) CTG(Leu161) to CTA(Leu) CCT(Pro369) to CCC(Pro)
320SHU022	**ATG(Met142) to TTG(Leu)** **GCG(Ala164) to GTG(Val)** ATC(Ile283) to GTC(Val) CAG(Gln524) to CGG(Arg)	GCG(Ala16) to GCA(Ala) GCA(Ala24) to GCC(Ala)

exhibited significant improvement of canthaxanthin percentage yields. The mutants, 320SHU019, 320SHU001 and 320SHU017, showed the highest percentage of canthaxanthin yields of approximately 90% (Table 2) in *E. coli* WS210. This novel in vitro recombination technique was an efficient method to create recombinant DNA products. The contamination of the parent templates in the library made by this method is undetectable.

The mutations occurred in the first-round error-prone PCR mutants and the second-round in vitro recombination mutants were analyzed by DNA sequencing (Table 3). All mutants contained amino acid substitutions in addition to silent mutations. Some of the mutations were observed in many mutants, such as ATG(Met142) to TTG(Leu), GCG(Ala164) to GTG(Val), ATC(Ile283) to GTC(Val), CGG(Arg519) to TGG(Trp), and CAG(Gln524) to CGG(Arg) or CTG(Leu). In particular, two mutations (Met142 to Leu and Ala164 to Val) were found in many of the recombined mutants exhibiting particularly high yields of canthaxanthin, namely, 320SHU001, 320SHU008, 320SHU016, 320SHU017, 320SHU019, and 320SHU022. These two mutations appeared to be inherited from the 320M4020 parent during in vitro recombination. The repeated isolation of the identical mutations in the improved *crtO* mutants suggested that these sites, and possibly the regions around these sites, were important for improving the *crtO*'s activity for canthaxanthin production.

3.3. Further improvement of canthaxanthin production in Methylomonas by increased gene expression

Two best mutants from the first round of error-prone PCR mutagenesis (320M4019 and 320M4009) and three best mutants from the second round of in vitro recombination (320SHU019, 320SHU001 and 320SHU017) were selected for analysis of canthaxanthin production in *Methylomonas*. The broad host range plasmids expressing the improved *crtO* genes together with the controls were conjugated into *Methylomonas* strain MWM1200. Table 4 shows that the performance of these mutant *crtO* genes has been greatly improved when compared with the starting gene. However, the percentage yield of canthaxanthin in *Methylomonas* was still below 50%, even for the best recombined mutants (Table 4). Several possible reasons could be accounted for the lower percentage yield of canthaxanthin in *Methylomonas*. For example, the intracellular microenvironment was different between *E. coli* cells and *Methylomonas* cells. The gene expression and production conditions were not optimized for *Methylomonas*.

Table 4
Analysis of canthaxanthin produced in *Methylomonas* MWM1200 expressing the selected CrtO mutants

Strain	Percentage yield of canthaxanthin[a]
CrtO320 (starting gene)	18
320M4009	28
320M4019	39
320SHU001	49
320SHU017	42
320SHU019	45
CrtW307 (control)	78

[a]Percentage yield of canthaxanthin in the total carotenoids. The remaining carotenoids in the cells were β-carotene and echinenone.

Since the percentage yield of canthaxanthin for some of the recombined mutants has reached ~90% in the high titer *E. coli*, the further improvement to increase canthaxanthin percentage yield of these mutant genes needs to be carried out in *Methylomonas*.

Mutant 320SHU001 showed the highest canthaxanthin percentage yield in *Methylomonas* (~49%) and this mutant gene *crtO** was chosen for further optimization in *Methylomonas* by increasing its gene expression. We observed earlier (unpublished data) that expression of the *crt* genes as influenced by the order of the genes in an operon could significantly change carotenoids profile. In the isolated mutant 320SHU001, the *crtO** gene was expressed as the second gene downstream of *crtE* in the operon (*crtEO*YIB*). Plasmid pDCQ354 was constructed by cloning the *crtO** as the first gene in the operon *crtO*EidiYIB*. Another β-carotene synthesis gene cluster containing an isoprenoid gene *idi* was used in pDCQ354, since the *idi*-containing β-carotene synthesis gene clusters exhibited higher β-carotene synthesis than the *idi*-lacking β-carotene synthesis gene clusters in *E. coli* (Sedkova et al., 2005). *E. coli* WS210 containing pDCQ354 produced ~99% of carotenoids as canthaxanthin. *Methylomonas* MWM1200 containing pDCQ354 produced ~93% of carotenoids as canthaxanthin. By combining improvement of gene structure and gene expression, we demonstrated production of >1000 ppm of canthaxanthin at 93% specificity in *Methylomonas* using a CrtO-type of β-carotene ketolase, compared to less than 18% canthaxanthin in the starting strain.

Considerable progress has been made to increase titers of carotenoids produced in heterologous hosts such as *E. coli* and *Methylomonas* (Alper et al., 2005a, b; Sharpe et al., 2007). The improved CrtO described here would be useful for making high titers of ketocarotenoids such as canthaxanthin in those hosts.

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