Available online at www.sciencedirect.com
ScienceDirect
Metabolic Engineering 8 (2006) 523–531
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Engineering a β-carotene ketolase for astaxanthin production

Luan Tao, Jolanta Wilczek, J. Martin Odom, Qiong Cheng*

Biological and Chemical Sciences and Engineering, Central Research and Development, E. I. DuPont de Nemours Inc., Wilmington, DE 19880-0328, USA

Received 13 April 2006; received in revised form 1 June 2006; accepted 12 June 2006
Available online 27 June 2006

Abstract

A new β-carotene ketolase gene (*crtW*) was cloned from an environmental isolate *Sphingomonas* sp. DC18. A robust and reliable color screen was developed for protein engineering to improve its activity on hydroxylated carotenoids for astaxanthin production. Localized random mutagenesis was performed on the *crtW* gene including the upstream ribosomal binding site (RBS). Six mutations (H96L, R203W, A205 V, A208 V, F213L and A215 T) in the *crtW* gene were isolated multiple times that showed improved astaxanthin production. These mutations were localized near the conserved histidine motifs, which were proposed for binding iron required for enzymatic activity. Combination of two of the mutations (R203W/F213L) further improved astaxanthin production. One mutation at the RBS (a438t) was shown to have additional effect on improving astaxanthin production. Most of the mutants still retained high activity on β-carotene, however, the F213L single mutant and the R203W/F213L double mutant that yielded the highest improvement for astaxanthin production showed decreased activity for canthaxanthin production.

Keywords: β-carotene ketolase; Astaxanthin; Adonixanthin; Zeaxanthin; Ribosomal binding site; Error-prone PCR

1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-caroten-4,4′-dione) is the most common carotenoid naturally present in the marine fish and shellfish (Miki et al., 1982) and has been widely used as a feed supplement in poultry and aquaculture industries. Astaxanthin also exhibits diverse biological activities such as strong antioxidant activity (Nishikawa et al., 2005), anti-photoaging effect (Arakane, 2002) and enhancement of immune responses (Chew and Park, 2004). Its application is emerging in pharmaceutical and personal care industries. Most of the industrially used astaxanthin is synthesized chemically, although some are derived from biological sources such as yeast *Xanthophyllomyces dendrorhous* (previously as *Phaffia rhodozyma*) (Visser et al., 2003) and green algae *Haematococcus pluvialis* (Guerin et al., 2003). Astaxanthin produced recombinantly from bacteria provides another competitive source for these applications.

Astaxanthin biosynthesis has been well studied in marine bacteria (Misawa et al., 1995; Yokoyama and Miki, 1995). The β-carotene (β,β-carotene) precursor was synthesized from farnesyl pyrophosphate by sequential reactions catalyzed by four enzymes encoded by *crtEBIY* genes. The rate-limiting step for astaxanthin biosynthesis was the subsequent oxygenation reactions from β-carotene to astaxanthin (Fig. 1), which were catalyzed by 4,4′-β-ionone ring ketolase (CrtW, also known as β-carotene ketolase) and 3,3′-β-ionone ring hydroxylase (CrtZ, also known as β-carotene hydroxylase). Both CrtW and CrtZ could catalyze oxygenation of the respective positions on one or both of the β-ionone rings regardless of whether the other enzyme acted first. Consequently, eight intermediates could be generated which affected astaxanthin conversion as measured by the percentage of astaxanthin produced relative to the total carotenoid content. In the native *Agrobacterium aurantiacum* (reclassified as *Paracoccus* sp. N81106), astaxanthin contributed to 19.5% of total carotenoids. In the native *Paracoccus marcusi*, astaxanthin contributed to 11.5% of total carotenoids. Adonixanthin (3,3′-dihydroxy-β,β′-caroten-4-one) was accumulated as

*Corresponding author. Fax: +1 302 695 1829.
E-mail address: qiong.cheng@usa.dupont.com (Q. Cheng).

1096-7176/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2006.06.001

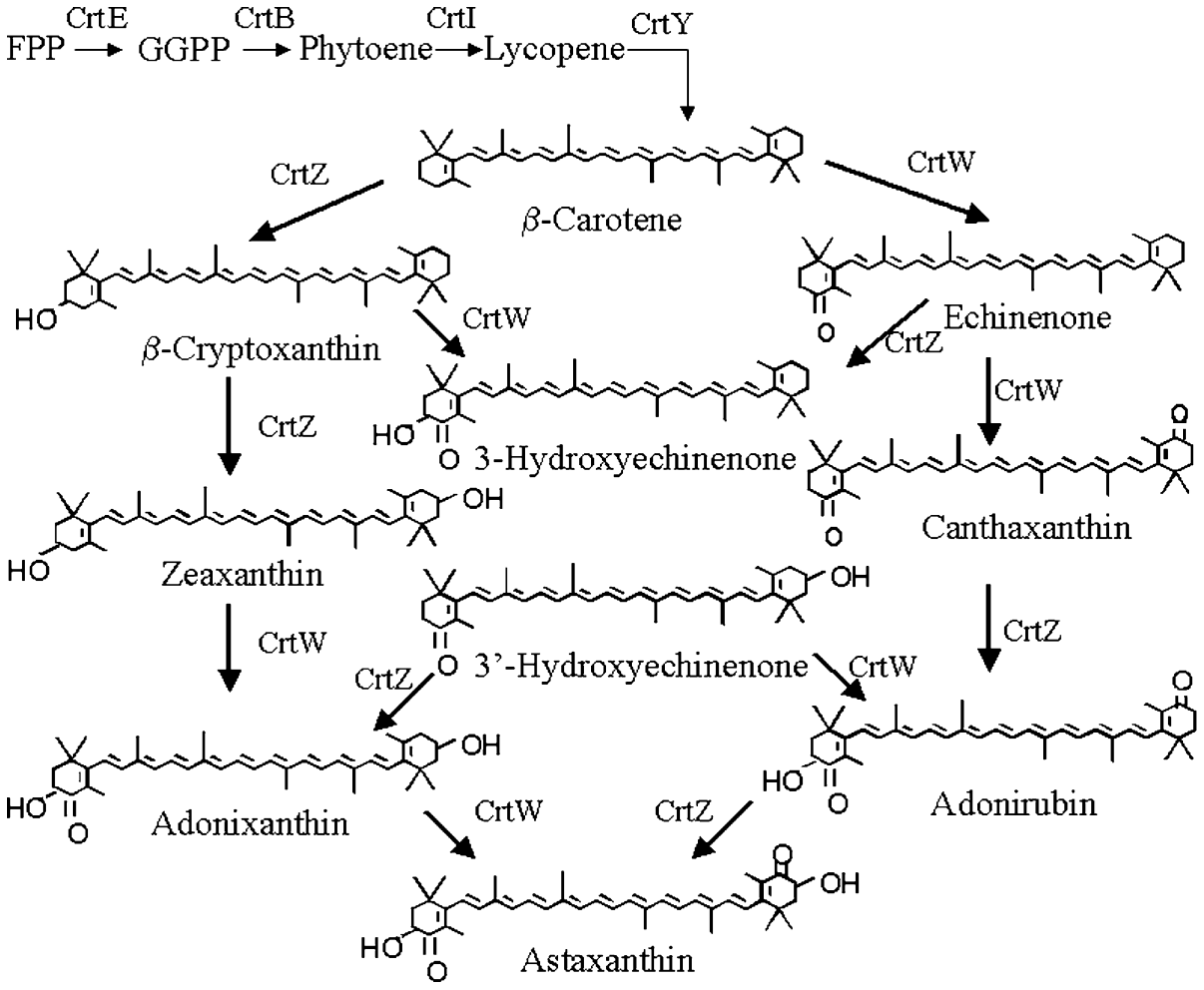

Fig. 1. Illustration of possible pathway intermediates in the synthesis of astaxanthin from β-carotene via the 4,4′-β-ionone ring ketolase (CrtW) and the 3,3′-β-ionone ring hydroxylase (CrtZ) reactions.

the major carotenoid in both bacteria. It appeared that the CrtW activity to further ketolate adonixanthin to astaxanthin was rather poor and created this bottleneck of conversion.

The CrtW and CrtZ enzymes from different bacterial sources were also characterized in vitro and showed different activity and substrate specificity (Fraser et al., 1997). The CrtZ from *Erwinia uredovora* (reclassified as *Pantoea ananatis*) showed high activity on β-carotene for producing zeaxanthin (β,β-carotene-3,3′-diol), and the CrtZ from *Alcaligenes* sp. PC-1 (reclassified as *Paracoccus* sp. PC-1) showed high activity on canthaxanthin (β,β-carotene-4,4′-dione) for producing astaxanthin. The CrtW enzymes efficiently introduced keto groups to β-carotene for canthaxanthin production, however, most of them exhibited limited activity towards hydroxylated carotenoids such as zeaxanthin and adonixanthin for producing astaxanthin. The in vitro activity data was consistent with the accumulation of adonixanthin observed in vivo. A recently isolated CrtW from *Brevundimonas* sp. SD212 showed higher activity on hydroxylated carotenoids (Choi et al., 2005).

In this article, we report the use of a protein engineering approach to improve the CrtW isolated from *Sphingomonas* sp. DC18 for astaxanthin production. We isolated more than forty CrtW mutants that produced more astaxanthin and less intermediates of hydroxylated carotenoids. Astaxanthin was produced at close to 90% of the total carotenoids in the best mutant comparing to 14% of astaxanthin produced in the wild type control.

2. Materials and methods

2.1. Strain isolation

Strain DC18 was isolated from a Pennsylvania stream. Serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of the aqueous sample were plated onto large 245×245 mm 1.5% agar plates with basal medium enriched with tryptone and yeast extract. The components of the basal medium were (in a liter): NH_4Cl 0.8 g, KH_2PO_4 0.5 g, $MgCl_2 \cdot 6H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $NaNO_3$ 1.3 g, Na_2SO_4 0.5 g. The basal medium was supplemented with solution 1 (10 ml per 1 litre). The components of the stock solution 1 were (in a

liter): nitrilotriacetic acid 12.8 g, $FeCl_2 \cdot 4H_2O$ 0.3 g, $CuCl_2 \cdot 2H_2O$ 0.0254 g, $MnCl_2 \cdot 4H_2O$ 0.1 g, $CoCl_2 \cdot 6\ H_2O$ 0.312 g, $ZnCl_2$ 0.1 g, H_3BO_3 0.01 g, $Na_2MoO_4 \cdot 2H_2O$ 0.01 g, $NiCl_2 \cdot 6H_2O$ 184 g. The medium was enriched with tryptone at 10 g/L and yeast extract at 5 g/L. Media pH was adjusted to 7. The plates were incubated at room temperature and single colonies were streaked twice onto the same plates. One which formed orange colonies was designated as strain DC18, which later was typed as *Sphingomonas* sp. DC18.

2.2. Library construction and screening

A small insert library was constructed from genomic DNA of strain DC18 by random shearing method. Genomic DNA of DC18 was sheared by passing through a 29G1/2 insulin syringe (Becton Dickinson, Franklin Lakes, NJ) about 300 times and separated on a 0.8% agarose gel. The 4–6 kb fraction was excised from the gel and extracted using Qiagen MinElute Gel-Extraction kit. The ends of the extracted DNA were repaired using Lucigen DNA Terminator Repair kit. The repaired DNA inserts were ligated to pEZseq vector using pEZSeq Blunt Cloning kit (Lucigen, Middletown, WI). The ligation mixture was electroporated into freshly prepared competent cells of *E. coli* 10 G containing a β-carotene-producing plasmid pDCQ329 (Sedkova et al., 2005). Transformants were plated on LB plates with 100 μg/ml ampicillin and 50 μg/ml kanamycin. The positive clones were identified as orange colonies from the background of yellow colonies.

2.3. Construction of a zeaxanthin reporter plasmid

An *E. coli* strain producing zeaxanthin from pDCQ395 was used as the reporter strain. Zeaxanthin synthesis plasmid pDCQ395 was constructed by cloning the β-carotene hydroxylase gene *crtZ* from *Pantoea agglomerans* DC404 upstream of the β-carotene producing plasmid pDCQ330, which contained the *crtEidiYIB* genes from DC404 (Sedkova et al., 2005). The DC404 *crtZ* was amplified with primers 5′-ACTAGTAAGGAGGAATAAACCATGCTTGCGTT GTGGAATACCG-3′ and 5′-TCTAGAGCCTAGGTTA TTTCCGGCGCGAAG-3′. The DNA fragment containing the *crtZ* was digested with SpeI and XbaI, and cloned into the SpeI site upstream of the *crtEidiYIB* genes in pDCQ330 resulting in the zeaxanthin-producing plasmid pDCQ395.

2.4. Site-directed mutagenesis

The *crtW* gene from DC18 was cloned downstream of the *trc* promoter on pTrcHis2-TOPO vector (Invitrogen). In order to have a better visual screening, the expression of the *crtW* on this plasmid pDCQ351 was reduced by mutating the first nucleotide of the −35 region TTGACA of the *trc* promoter from T to C using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used were 5′-CTGAAATGAGCTGCTGACAAT-TAATCATCCGGCTCG-3′ and 5′-CGAGCCGGATGA TTAATTGTCAGCAGCTCATTTCAG-3′. Plasmid pDC Q351M containing the wild type DC18 *crtW* gene expressed from the mutated *trc* promoter was confirmed by sequencing.

A double mutant containing both F213L and R203W mutations was constructed by introducing the targeted R203W mutation in a single F213L mutant using the Stratagene's site-directed mutagenesis kit. The primers used were: 5′-AATGCCCGCAGCAACGGCTGGCCAT GGCTGGCGTCGCTGGCGAC-3′ and 5′-GTCGCCAG CGACGCCAGCCATGGCCAGCCGTTGCTGCGGGC ATT-3′. Plasmid pDCQ425 containing the R203W/F213L double mutation was confirmed by sequencing.

The a438t ribosomal binding site (RBS) mutation was introduced into the upstream region of the mutated *crtW* gene in the MM2 mutant. The primers used were 5′-TGGCCCTTTCTAGAAAGGAGGAATTAACCATG ACCGTCGATCACGAC-3′ and 5′-GTCGTGATCGAC GGTCATGGTTAATTCCTCCTTTCTAGAAAGGGC CA-3′. The plasmid pDCQ426 containing the a438t RBS mutation in addition to the original mutations in MM2 was confirmed by sequencing.

2.5. Error-prone PCR mutagenesis

Error-prone PCR was performed with the NcoI-EcoRI fragment on pDCQ351M, which include the *crtW* gene and its upstream ribosomal binding site (RBS). PCR reaction was performed as previously described (Tao et al., 2005a). The purified PCR products from reactions containing different amount of $MnCl_2$ were combined and digested with NcoI and EcoRI. The digested error prone PCR product was gel purified and ligated to NcoI/EcoRI sites of pDCQ351M to replace the wild-type *crtW* gene and its RBS. The ligated DNA was electroporated into *E. coli* 10G (pDCQ395) reporter cells. The transformed cells were spread on LB agar containing 50 μg/ml kanamycin and 100 μg/ml ampicillin, and incubated at 30 °C for 2–3 days. Colonies that showed orange/red color indicating improved β-carotene ketolase activity were isolated.

2.6. Carotenoids analysis

DC18 was grown at 30 °C shaking 1–2 days in 100 ml of the same medium as described for the strain isolation. Cells were pelleted by centrifugation at 4000*g* for 15 min, and cell pellets were extracted twice with 10 ml acetone, which removed all the pigments in the cell pellets. The extraction was dried under nitrogen and redissolved in 1–2 ml of acetone. The extraction was filtered with an Acrodisc® CR25 mm syringe filter (Pall Corporation, Ann Arbor, MI). It was then concentrated in 0.1 ml 10% acetone + 90% acetonitrile for LC/MS analysis. Carotenogenic *E. coli* cells were grown either in flasks or 24-well blocks at

30 °C shaking 1–2 days. Cells were pelleted and extracted twice with 50% acetone + 50% methanol. The extraction was filtered and loaded for HPLC using an Agilent Series 1100 LC/MSD (Agilent, Foster City, CA). The HPLC-MS method used for analysis of the carotenoids was described previously (Tao et al., 2005b). The synthetic standards of canthaxanthin, zeaxanthin, adonixanthin, and astaxanthin were purchased from CaroteNature (Lupsingen, Switzerland).

2.7. Nucleotide accession number

The nucleotide sequence of the 4,4′-β-ionone ring ketolase gene (*crtW*) from *Sphingomonas* sp. DC18 has been deposited in GenBank under accession number DQ400932.

3. Results

3.1. Cloning of a β-carotene ketolase gene from an environmental isolate

Strain DC18 was an orange-pigmented bacterium isolated from a Pennsylvania stream. The near-complete (1291 bp) 16S rDNA sequence of DC18 was determined and it showed high homology to those of *Sphingomonas* strains, with the top hit as 98% similar to *Sphingomonas melonis*. This orange isolate was designated *Sphingomonas* sp. DC18. Carotenoid analysis suggested that DC18 produced ketocarotenoids. The absorption spectrum of the major carotenoid in DC18 was round-shaped at 464 nm (λ_{max}: 464). LC-MS analyses determined the molecular weight of the major carotenoid in DC18 to be 614. The major carotenoid in DC18 was predicted to be dihydroxy-adonixanthin. The properties we determined for the major carotenoid in DC18 (λ_{max}: 464: m/e: 614) were consistent with what reported in the literature for 2,3,2′,3′-tretrahydroxy-β,β′-caroten-4-one (dihydroxy-adonixanthin) (Kleinig et al., 1977; Yokoyama et al., 1996). Strain DC18 was thus used as a source for cloning a β-carotene ketolase gene.

The β-carotene ketolases catalyze the conversion from β-carotene to canthaxanthin, which could be identified by turning β-carotene-producing *E. coli* cells from yellow to orange. Several orange colonies were identified from approximately 100,000 *E. coli* transformants of the small insert library of DC18. HPLC analysis showed that ketocarotenoids (canthaxanthin and echinenone) were produced in the positive *E. coli* clones. The insert on one of the positive clones was sequenced and confirmed that it contained an intact β-carotene ketolase gene (*crtW*). The gene encoding the *crtW* from DC18 showed homology to β-carotene ketolases from many organisms, with the highest hit as 57% amino acid identity and 70% amino acid similarity to the β-carotene C4 oxygenase from *Brevundimonas aurantiaca* (gi|33439708|gb|AAN86030).

The *crtW* gene from DC18 was cloned downstream of the *trc* promoter on pDCQ351. When this expression clone was transformed into a β-carotene-accumulating *E. coli* strain containing pDCQ330 (Sedkova et al., 2005), orange transformants were obtained and they produced exclusively canthaxanthin. This clearly demonstrated the ketolase function of the new *crtW* gene from DC18.

3.2. Design of a color screening based on decreased expression of the β-carotene ketolase gene in E. coli

Although most ketolases are very active on β-carotene to make canthaxanthin, they have limited activity on hydroxylated carotenoids such as zeaxanthin and adonixanthin to make astaxanthin (Fraser et al., 1997). CrtW mutants for higher astaxanthin production could be isolated by screening for orange or orange/red colonies in a zeaxanthin reporter strain. When pDCQ351 expressing the wild type *crtW* from the *trc* promoter was transformed into the zeaxanthin reporter *E. coli* containing pDCQ395, cells turned orange indicating that the background color from the wild type gene was a little too high for visual screening. Analysis showed that the cells produced a mixture of carotenoids containing 33% astaxanthin, 51% adonixanthin and 16% zeaxanthin. In order to have a better visual screening and to isolate more active CrtW mutants, the expression of the *crtW* was reduced by changing the first nucleotide of the −35 region (TTGACA) of the *trc* promoter from T to C by site-directed mutagenesis. The expression clone pDCQ351M contained the wild type *crtW* expressed from the mutated *trc* promoter. When pDCQ351M was transformed into the zeaxanthin reporter, cells were yellow which allowed screening of improved *crtW* mutants by looking for colonies turning orange or orange/red. Analysis showed that these cells produced a mixture of carotenoids containing 12% astaxanthin, 51% adonixanthin and 34% zeaxanthin.

3.3. Isolation of mutant β-carotene ketolase genes that increased astaxanthin production

Error-prone PCR was performed with the NcoI-EcoRI fragment on pDCQ351 M, which include the *crtW* gene and its upstream RBS. Approximately 100,000 colonies were obtained from this *crtW* mutagenesis library. Forty-eight orange or orange/red colonies were isolated. All of these mutants showed higher astaxanthin production than the wild type *crtW* control. The highest was the MM1 mutant that produced 83% astaxanthin comparing to 14% astaxanthin produced by the wild type control.

These mutants were sequenced and the mutations were localized in several hot spots. Among all the 48 sequenced mutants, 40 mutants contained mutations that occurred more than once (Table 1). Some were isolated as the single mutants, and some were isolated as the mutants combined with other mutations. The F213L mutation was isolated 11 times. The R203W mutation was also isolated 11 times. The A215T mutation was isolated seven times. The A205V mutation was isolated six times. The A208V mutation was

Table 1
Sphingomonas sp. DC18 *crtW* mutations isolated more than once from the mutagenesis library that increased astaxanthin production

Mutation[a]	RBS[b]	Additional mutation	Silent mutation	AST%
Control				14
F213L(t1079c)[c]				67–74
F213L(c1081g)				72
F213L(c1081a)				71
F213L(t1079c)			A164A(g934a)	70
F213L(t1079c)			R128R (c826t)	69
F213L(t1079c)			A50A(g592a)	76
F213L(t1079c)			V147V(g883a)	71
F213L(t1079c)		N199S(a1038g)	V79V(c679t), R184R(c994t)	71
R203W(c1049t)[d]				51
R203W(c1049t)			L165L(t935c)	51
R203W(c1049t)			P70P (c652t)	50
R203W(c1049t)			T108T (c766t)	51
R203W(c1049t)			A28A(g526a)	54
R203W(c1049t)		R33Q(g540a)	S198S(c1036t)	57
R203W(c1049t)	t424c	L165V(t935g)		56
R203W(c1049t)	a436g	K246R(a1179g)		49
R203W(c1049t)[e]		A193T(g1019A)	N195N(t1027c)	40
R203W(c1049t)		A144V(c873t, g874t)	L47L (c583a)	54
A215T(g1085a)				53
A215T(g1085a)			G85G (c697t)	50
A215T(g1085a)			T2T (c448a)	70
A215T(g1085a)		T131A(a833g)	P106P (g760a)	69
A215T(g1085a)		W225R(t1115c)	A20A (g502a)	59
A215T(g1085a)		V30M(g530a), T131A(a833g)		62
A215T(g1085a)		V147E(t882a)		67
A205V(c1056t)				44
A205V(c1056t)			D6D(c460t), P73P (c661t)	52
A205V(c1056t)			L165L (t935c), P202P (a1048g)	66
A205V(c1056t)		V154A(t903c)		51
A205V(c1056t)		T38A(a554g), L41F(c563t), A74S(g662t), S245G(a1175g)	A60A (g622a)	46
A205V(c1056t)		R203Q(g1050a)	H96H (t730c), S121S(c805a)	35
A208V(c1065t)			G55G (c607a)	43
A208V(c1065t)		I9V(a467g)		44
A208V(c1065t)[f]	a438t	H241R(a1164g)	V59V (c619g)	83
H96L(a729t)	t437c		G55G (c607t)	64
H96L(a729t)		A193E(c1020a)	L151L (c893t)	43

[a]The nucleotide change for the corresponding amino acid change is shown in parenthesis. The number indicates the position of the change. The letter before the number indicates the wild type residue, and the letter after indicates the mutation residue.

[b]The C441 deletion in the RBS was present in all the mutants and the control, which is not shown in this table. All the mutants and the control also contain −35 trc promoter *t*→c change, which is not shown in this table.

[c]The F213L (t1079c) mutants were isolated four times.

[d]The R203W(c1049t) mutants were isolated twice.

[e]This R203W mutant containing additional A193T mutation and silent N195N mutation was designated as mutant MM2.

[f]This A208V mutant containing additional a438t mutation at the RBS and H241R mutation and silent V59V mutation was designated as mutant MM1.

isolated three times. The H96L mutation was isolated twice. The H96L mutation was close to the second conserved His motif in the middle of the CrtW protein (Fig. 2). All the other mutations that were isolated multiple times were clustered at the carboxyl terminal region of the CrtW protein near the third conserved His motif. The three histidine motifs were shown to be conserved in membrane-bound iron-requiring enzymes such as fatty acid desaturases (Dyer et al., 2002; Shanklin et al., 1994). They were proposed to be involved in Fe^{2+} binding and essential for catalytic activity of the enzymes.

One mutant of each mutation was selected for further analysis. The selected R203W, A205 V, F213L or A215T mutant contained only the indicated single mutation. The selected A208V mutant contained an additional silent mutation at glycine 55. The selected H96L mutant also contained an additional silent mutation at glycine 55 as well as a RBS mutation a437t. These mutants together with the control strain containing the wild type *crtW* on pDCQ351M were grown for 8 h or 48 h at 30 °C for carotenoid analysis (Fig. 3). The F213L single mutation after 48 h showed the largest effect on improving astaxanthin production (~77% astaxanthin). The selected A208V mutant containing the additional G55 silent mutation produced ~56% astaxanthin. The MM1 mutant contained several mutations in addition to the A208V

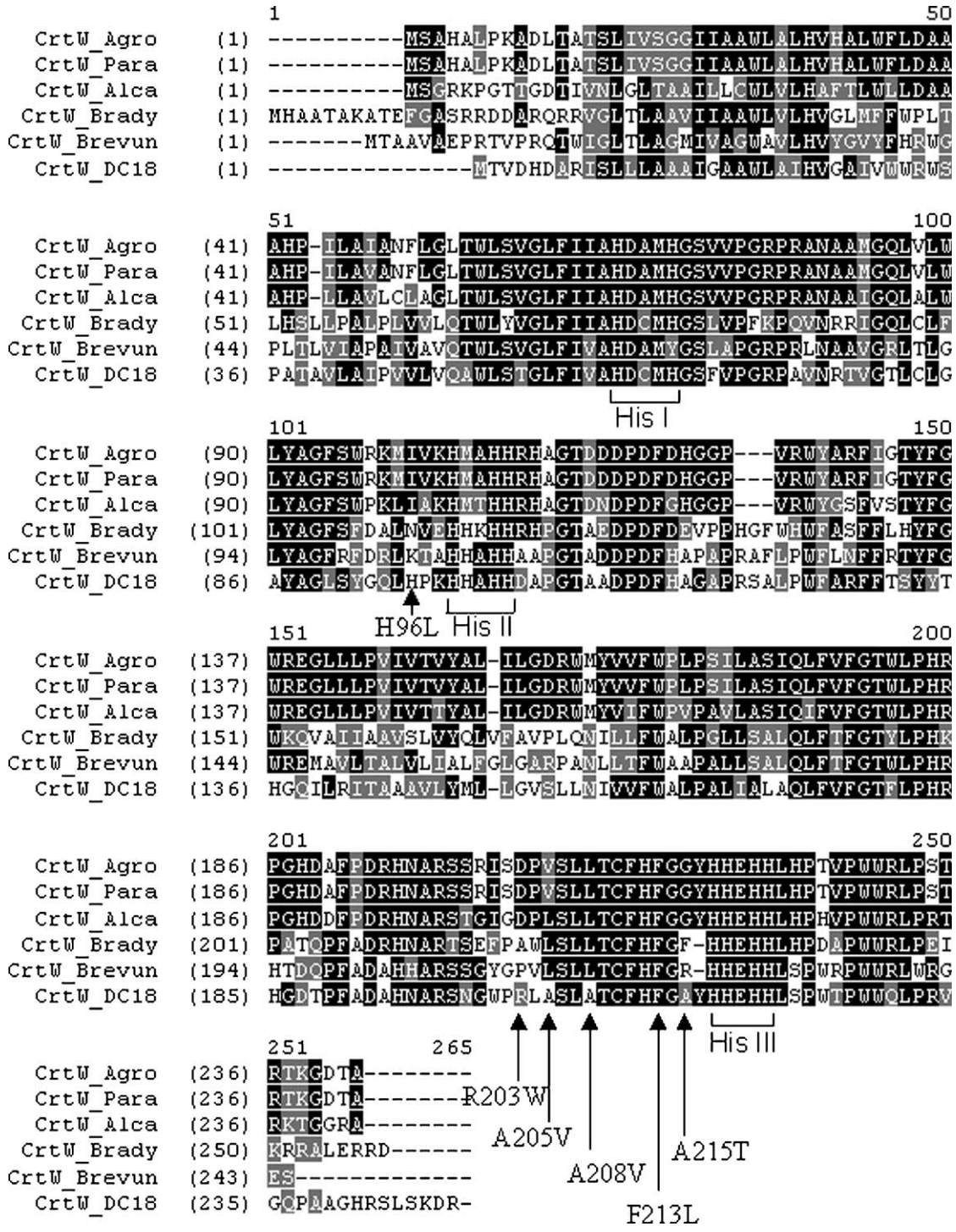

Fig. 2. Multiple sequence alignment of the 4,4′-β-ionone ring ketolases (CrtW) from different bacterial species. The accession number for the CrtW from *Agrobacterium aurantiacum* (reclassified as *Paracoccus* sp. N81106) is D58420. The accession number for the CrtW from *Paracoccus marcusii* is Y15112. The accession number for the CrtW from *Alcaligenes* sp. (reclassified as *Paracoccus* sp. PC1) is D58422. The accession number for the CrtW from *Bradyrhizobium* sp. is AF218415. The accession number for the CrtW from *Brevundimonas aurantiaca* is AY166610. The accession number for the CrtW from *Sphingomonas* sp. DC18 is DQ400932. The three conserved histidine motifs in the CrtW proteins were indicated. Locations of the isolated mutations in the DC18 CrtW were also marked.

mutation in the *crtW* gene (A208 V, H241R and V59 V) as well as a mutation at the RBS (a438t). The high astaxanthin in MM1 (83%) was most likely a combinational effect of the beneficial gene mutation(s) and the possible beneficial RBS mutation.

3.4. Beneficial RBS mutation had additive effect on increasing astaxanthin production

To confirm that the mutations in the *crtW* gene and/or the RBS site in mutant MM1 contributed to the phenotype

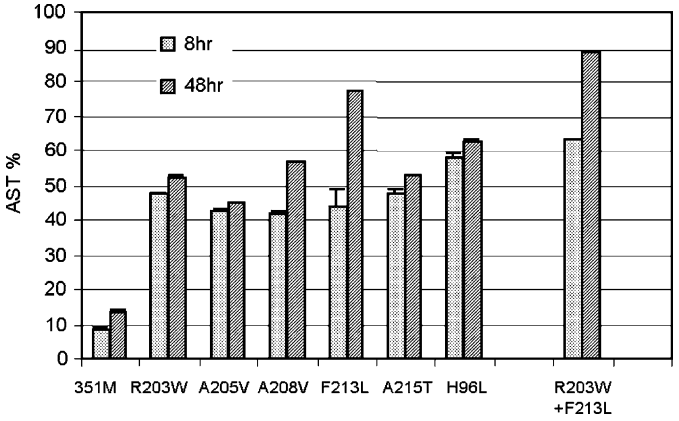

Fig. 3. Astaxanthin conversion from zeaxanthin-accumulating *E. coli* strains containing the wild type DC18 *crtW* (351M) and the mutant derivatives all expressed from the reduced *trc* promoter. Carotenoids were extracted from the cultures after 8- and 48-h growth at 30 °C. The means and standard deviations were calculated from triplicate data.

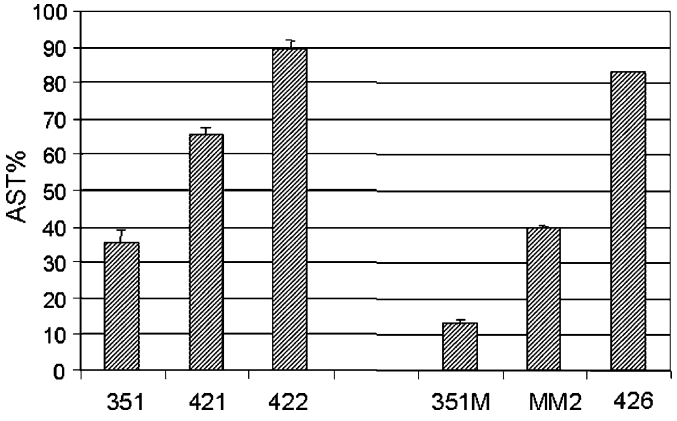

Fig. 4. Effect of the a438t RBS mutation upstream of the CrtW mutant derivatives on astaxanthin conversion in zeaxanthin-accumulating *E. coli* strains. The first group of strains contained the wild type *crtW* (351), the MM1 mutant *crtW* (421) and the MM1 mutant *crtW* with the RBS mutation (422) expressed under the wild type *trc* promoter. The second group of strains contained the wild type *crtW* (351M), the MM2 mutant *crtW* (MM2) and the MM2 mutant *crtW* with the RBS mutation (426) expressed under the reduced *trc* promoter. The means and standard deviations were calculated from triplicate data.

of enhanced astaxanthin production, the mutant *crtW* gene of MM1 was subcloned with the wild type RBS (pDCQ421) or the mutant RBS (pDCQ422) into the pTrcHis2 vector with the wild type *trc* promoter. Comparing with the *E. coli* containing pDCQ351 expressing the wild type *crtW* gene under the wild type *trc* promoter, *E. coli* cells containing pDCQ421 or pDCQ422 both showed higher astaxanthin production (Fig. 4). Combining the *crtW* mutations with the RBS mutation on pDCQ422 yielded higher astaxanthin than pDCQ421 with only the *crtW* mutations. This 91% astaxanthin production was even higher than that from the original MM1 mutant expressing the same mutations under the reduced *trc* promoter. This confirmed that mutations in MM1 contributed to the enhanced astaxanthin production. The a438t mutation in the RBS had an additional positive effect on astaxanthin production.

To determine if the a438t RBS mutation could exert its additional positive effect when it was combined with another gene mutation, the a438t RBS mutation was introduced into the upstream region of the *crtW* gene in mutant MM2 containing three gene mutations (R203W, A193T, N195N). The plasmid pDCQ426 contained the a438t RBS mutation in addition to the original mutations in MM2. It showed twice as much of astaxanthin production in zeaxanthin-producing *E. coli* cells as that from the original MM2 mutant (Fig. 4). This demonstrated that the beneficial effect of a438t mutation is rather broadly applicable, which likely increased protein expression through enhanced efficiency for translation.

3.5. Combination of different beneficial crtW mutations to further increase astaxanthin production

Both F213L and R203W mutations were isolated with high frequencies. Single F213L mutant showed about 77% astaxanthin conversion. Single R203W mutant showed about 52% astaxanthin conversion. A double mutant containing both F213L and R203W mutations was constructed by introducing the targeted R203W mutation in a single F213L mutant. Plasmid pDCQ425 containing the R203W/F213L double mutation was transformed into the *E. coli* zeaxanthin reporter strain. These *E. coli* cells showed 88% astaxanthin conversion, which was higher than the individual mutants (Fig. 3). This demonstrated that higher astaxanthin production could be achieved by combining different beneficial mutations in the CrtW.

3.6. Assay of the CrtW mutants for canthaxanthin production

All the isolated DC18 ketolase mutations enhanced astaxanthin production in the zeaxanthin-accumulating *E. coli* strain. To determine how these ketolase mutations affected their activity towards β-carotene for canthaxanthin production, plasmids were isolated from selected mutant strains and the parent control and transformed into *E. coli* accumulating β-carotene (Fig. 5). Most of the strains including the parent control showed high canthaxanthin production. They produced ~90% canthaxanthin after 8-h growth and ~98% canthaxanthin after 48-h growth. The only single mutant that showed decreased canthaxanthin production was F213L. It produced ~65% canthaxanthin after 8-h growth and ~85% canthaxanthin after 48-h growth. Similar lower amount of canthaxanthin production was also observed with the constructed double mutant R203W/F213L. It is interesting to note that the single mutant F213L and the double mutant R203W/F213L showed the highest astaxanthin production (Fig. 3), which produced about 6-fold as high as the parent control. However, they showed even less activity for canthaxanthin production than the parent control.

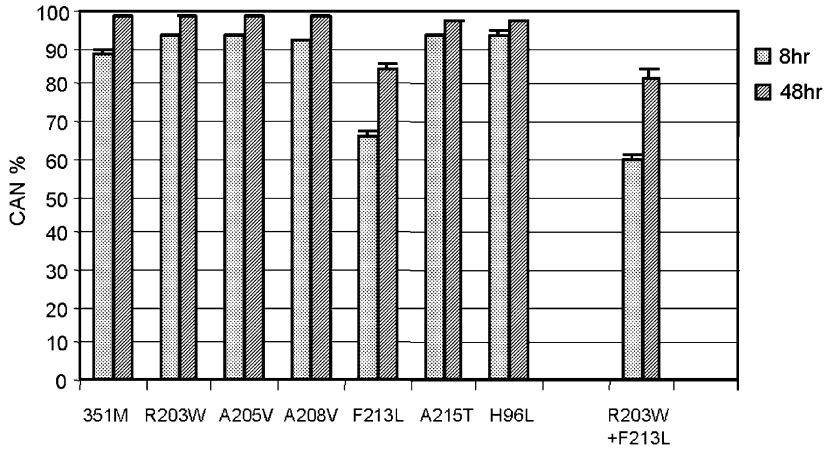

Fig. 5. Canthaxanthin conversion from β-carotene-accumulating *E. coli* strains containing the wild type DC18 *crtW* (351M) and the mutant derivatives all expressed from the reduced *trc* promoter. Carotenoids were extracted from the cultures after 8- and 48-h growth at 30 °C. The means and standard deviations were calculated from triplicate data.

4. Discussion

One challenge for astaxanthin production in bacteria and plants is the low astaxanthin conversion, which is mainly affected by the activities and balance of the CrtWZ enzymes. Protein engineering was applied here to improve the activity of the CrtW from *Sphingomonas* sp. DC18 on hydroxylated carotenoids, which appeared to be a bottleneck for astaxanthin synthesis. A robust color screen was developed with a zeaxanthin reporter strain, which used the *crtZ* gene from *Pantoea* for efficient zeaxanthin synthesis. The promoter for the *crtW* gene expression was tuned down so that the wild type CrtW produced predominantly hydroxylated carotenoids (zeaxanthin, λ_{max} 452 nm; adonixanthin, λ_{max} 464 nm). These yellow cells became orange or orange/red as more astaxanthin (λ_{max} 480 nm) was produced resulting from higher CrtW activity on hydroxylated carotenoids. This screen was quite reliable as more than 40 mutants converged to six common mutations isolated multiple times. Most of the mutants were independent isolates as they contained different nucleotide mutations or different secondary mutations.

All the isolated mutants produced more astaxanthin and less intermediates, which were hydroxylated carotenoids of zeaxanthin and adonixanthin. The total carotenoid titer in the wild type and all the mutants was similar. The higher amount of astaxanthin production was contributed by the higher astaxanthin conversion in the mutants. All the mutants contained at least one mutation in the *crtW* gene, some contained multiple mutations in the gene, and several contained additional mutations at the RBS. All the silent mutations in the gene did not appear to change codon usage preference significantly. Transcriptional expression of all characterized *crtW* mutants (H96L, R203W, A205 V, A208 V, F213L and A215 T) was similar or less than the wild type *crtW* gene by RT-PCR analysis (data not shown). It is suggested that the improved astaxanthin production in the mutants was mostly due to change of the CrtW properties rather than the increase of *crtW* gene expression, although in several cases that the additional RBS mutations may have contributed to the increase of protein translation. The change of the CrtW properties could be caused either by increase of enzyme kinetics, which improved the competition with CrtZ for β-carotene, or by higher substrate specificity of the CrtW enzyme for the 3-hydroxy carotenoids.

The β-carotene ketolases CrtW and the β-carotene hydroxylases CrtZ belong to the family of membrane-bound, non-heme, iron-containing oxygenases, which includes fatty acid desaturases, alkane hydroxylases and xylene monooxygenases (Fraser et al., 1997; Shanklin et al., 1994). This group of enzymes has been suggested to have a related protein fold with conserved hydrophobic transmembrane domains and histidine-rich motifs. The three histidine motifs (HX_3H, HX_2HH and HX_2HH) were involved in coordination of the binding of Fe^{2+}, a cofactor needed for enzyme activity. Each of the eight histidine residues in a rat Δ^9 desaturase was shown to be catalytically essential (Shanklin et al., 1994) that mutation to alanine abolished the activity. One of the CrtW mutations we isolated in this study that showed improved astaxanthin production was at histidine 96, which was three amino acid residues upstream of the first conserved histidine residue in the second His motif (HisII, Fig. 2). Interestingly, all other CrtW mutations we isolated multiple times that improved astaxanthin production were also clustered upstream of a His motif (HisIII). It is likely that the regions upstream of the conserved His motifs constitute to part of the activity domain of the enzyme.

The F213L mutation, which was three amino acid residues upstream of the HisIII motif, is worth noting. The F213L single mutant showed the highest improvement for astaxanthin production, however, it showed decreased activity than the wild type enzyme on β-carotene for canthaxanthin production. The F213L mutation appeared to be a dominant mutation since similar phenotype was

also observed in the double mutant R203W/F213L. It is likely that the F213L mutant had a change of substrate specificity, which more preferred zeaxanthin substrate for producing astaxanthin than β-carotene substrate for producing canthaxanthin. Use of error-prone PCR and directed evolution has previously been reported to change substrate specificity of carotenoid synthesis enzymes for synthesis of novel carotenoids (Schmidt-Dannert et al., 2000). The β-carotene ketolases from many other organisms all contained the conserved phenylalanine as the F213 in the CrtW of *Sphingomonas* DC18. This conserved phenylalanine was located at position 240 in the CrtW of *Brevundimonas* DC263 (GenBank accession number DQ309446) and at position 230 in the CrtW of *Algoriphagus* KK10202C (GenBank accession number DQ286432). The similar change of phenylalanine to leucine in the DC263 CrtW (F240L) and the KK10202C CrtW (F230L) did not show obvious increase in astaxanthin production as the F213L mutant of the DC18 CrtW did. It appeared that the surrounding amino acid residues and the overall protein structures affected the function of the conserved residues in these protein homologues. Without the guidance from protein crystal structure, random mutagenesis is a more efficient way to improve protein functions.

Acknowledgments

We are grateful to Raymond E Jackson and Catherine S Byrne for their expert assistance with RT-PCR analysis. We also thank Rick Ye for critical reading of the manuscript.

References

Arakane, K., 2002. Superior skin protection via astaxanthin. Carot. Sci. 5, 21–24.

Chew, B.P., Park, J.S., 2004. Carotenoid action on the immune response. J. Nutr. 134, 257S–261S.

Choi, S.K., Nishida, Y., Matsuda, S., Adachi, K., Kasai, H., Peng, X., Komemushi, S., Miki, W., Misawa, N., 2005. Characterization of beta -carotene ketolases, CrtW, from marine bacteria by complementation analysis in *Escherichia coli*. Mar. Biotechnol. (NY) 7, 515–522.

Dyer, J.M., Chapital, D.C., Kuan, J.C., Mullen, R.T., Turner, C., McKeon, T.A., Pepperman, A.B., 2002. Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung. Implications for the evolution of plant fatty acid diversity. Plant Physiol. 130, 2027–2038.

Fraser, P.D., Miura, Y., Misawa, N., 1997. In vitro characterization of astaxanthin biosynthetic enzymes. J. Biol. Chem. 272, 6128–6135.

Guerin, M., Huntley, M.E., Olaizola, M., 2003. Haematococcus astaxanthin: applications for human health and nutrition. Trends Biotechnol. 21, 210–216.

Kleinig, H., Heumann, W., Meister, W., Englert, G., 1977. Carotenoids of *Rhizobia*. I. New carotenoids from *Rhizobium lupini*. Helv. Chim. Acta 60, 254–258.

Miki, W., Yamaguchi, K., Konosu, S., 1982. Comparison of carotenoids in the ovaries of marine fish and shellfish. Comp. Biochem. Physiol. B 71, 7–11.

Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T., Miki, W., 1995. Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J. Bacteriol. 177, 6575–6584.

Nishikawa, Y., Minenaka, Y., Ichimura, M., Tatsumi, K., Nadamoto, T., Urabe, K., 2005. Effects of astaxanthin and vitamin C on the prevention of gastric ulcerations in stressed rats. J. Nutr. Sci. Vitaminol. (Tokyo) 51, 135–141.

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

Sedkova, N., Tao, L., Rouvière, E.P., Cheng, Q., 2005. Diversity of carotenoid synthesis gene clusters from environmental *Enterobacteriaceae* strains. Appl. Environ. Microbiol. 71, 8141–8146.

Shanklin, J., Whittle, E., Fox, B.G., 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33, 12787–12794.

Tao, L., Jackson, R.E., Cheng, Q., 2005a. Directed evolution of copy number of a broad host range plasmid for metabolic engineering. Metab. Eng. 7, 10–17.

Tao, L., Schenzle, A., Odom, J.M., Cheng, Q., 2005b. Novel carotenoid oxidase involved in biosynthesis of 4,4′-diapolycopene dialdehyde. Appl. Environ. Microbiol. 71, 3294–3301.

Visser, H., van Ooyen, A.J., Verdoes, J.C., 2003. Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*. FEMS Yeast Res. 4, 221–231.

Yokoyama, A., Miki, W., 1995. Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. FEMS Microbiol. Lett. 128, 139–144.

Yokoyama, A., Miki, W., Izumida, H., Shizuri, Y., 1996. New trihydroxy-keto-carotenoids isolated from an astaxanthin-producing marine bacterium. Biosci. Biotech. Biochem. 60, 200–203.