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ORIGINAL PAPER

Construction of the astaxanthin biosynthetic pathway in a methanotrophic bacterium *Methylomonas* sp. strain 16a

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Abstract *Methylomonas* sp. strain 16a is an obligate methanotrophic bacterium that uses methane or methanol as the sole carbon source. An effort was made to engineer this organism for astaxanthin production. Upon expressing the canthaxanthin gene cluster under the control of the native *hps* promoter in the chromosome, canthaxanthin was produced as the main carotenoid. Further conversion to astaxanthin was carried out by expressing different combinations of *crtW* and *crtZ* genes encoding the β-carotenoid ketolase and hydroxylase. The carotenoid intermediate profile was influenced by the copy number of these two genes under the control of the *hps* promoter. Expression of two copies of *crtZ* and one copy of *crtW* led to the accumulation of a large amount of the mono-ketolated product adonixanthin. On the other hand, expression of two copies of *crtW* and one copy of *crtZ* resulted in the presence of non-hydroxylated carotenoid canthaxanthin and the mono-hydroxylated adonirubin. Production of astaxanthin as the predominant carotenoid was obtained in a strain containing two complete sets of carotenoid biosynthetic genes. This strain had an astaxanthin titer ranging from 1 to 2.4 mg g^{-1} of dry cell biomass depending on the growth conditions. More than 90% of the total carotenoid was astaxanthin, of which the majority was in the form of *E*-isomer. This result indicates that it is possible to produce astaxanthin with desirable properties in methanotrophs through genetic engineering.

Keywords Methane · Methanotroph · Pathway engineering · Carotenoids · Canthaxanthin · Astaxanthin

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Introduction

Obligate methanotrophs are ubiquitous in nature and found in a variety of habitats including aquatic and terrestrial systems and phyllosphere of plants [12]. They thrive in different conditions such as thermophilic, low pH, and high salinity environments [31]. This group of organisms play an important role in the recycling of green house methane formed in wetlands, rice fields, tundra, and marine environments. Recently, methane emissions from terrestrial plants under aerobic conditions have been identified [16]. This study also reveals a previously unrecognized source of carbon for methanotrophs. In industrial applications, methanotrophs have an enormous potential in bioremediation and biodegradation of environmental pollutants due to the diverse activities of the methane monooxygenase [17, 27]. In addition, utilization of methane or methanol as the carbon source to produce commercial products has attracted interests. The most recent venture was the large-scale production of whole-cell proteins and protein-based products from *Methylococcus capsulatus* based on fermentation with natural gas as the feedstock (Norferm AS, Odense, Denmark). However, efforts to develop a methane-based bioprocess for the production of high value products by introducing new pathways through genetic engineering remain scarce. It

is the aim of this experiment to explore the possibility of constructing a biosynthetic pathway in methanotrophs to produce high value products such as canthaxanthin and astaxanthin.

Both canthaxanthin and astaxanthin are carotenoids that have been widely used commercially [13]. In aquaculture industry, they are applied as colorants in fish feed. Although astaxanthin mainly provides pigmentation in farmed fish, it has been found to be important for their proper growth and survival [30]. Carotenoids are also powerful antioxidants and they are used as supplements in nutraceutical industries. Benefits of astaxanthin to human health have been suggested [14]. Currently, a majority of the commercial astaxanthin is synthesized chemically. The natural form of this pigment is produced from the microalga *Haematococcus pluvialis* [11] and pink yeast *Xanthophyllomyces dendrorhous* [15]. Biosynthesis of carotenoids is derived from the isoprenoid pathway [4]. Genes, *crtE*, *crtY*, *crtI*, and *crtB* can be used to produce the β-carotene from farnesyl pyrophosphate [18–20]. The two β-ionone rings in β-carotene are subsequently modified by hydroxylases and ketolases to produce canthaxanthin and astaxanthin. In *X. dendrorhous*, a novel cytochrome-P450 enzyme is recently found to be responsible for both the ketolase and hydroxylase activities [2, 22]. On the other hand, in some bacterial systems, the β-carotene hydroxylase CrtZ introduces the hydroxyl groups on the β-ionone rings, while the β-carotene ketolase CrtW catalyzes the reaction of adding two keto-groups [6, 7, 19, 20]. Only the CrtW ketolase is necessary for the production of canthaxanthin, while a combination of both CrtZ hydroxylase and CrtW ketolase are required for the biosynthesis of astaxanthin. There are eight possible intermediates produced during the biosynthesis of astaxanthin (Fig. 1). Both enzymes have different substrate specificities towards these compounds [8, 9]. This potential imbalance can lead to the production of intermediate compounds in conjunction with astaxanthin As a result an adequate activity of both enzymes is required to achieve a high conversion of the starting β-carotene substrate to astaxanthin.

In this report, the biosynthetic pathway involved in canthaxanthin and astaxanthin biosynthesis was introduced in a methanotrophic bacterium *Methylomonas* sp. strain 16a. During construction and evaluation of different strains, one of the major challenges identified was the accumulation of undesirable intermediates that reduced the astaxanthin selectivity. This problem was overcome by expressing multiple copies of carotenoid biosynthetic genes. The final strain constructed had a stable phenotype and was capable of producing astaxanthin as the predominant carotenoid product. The result shows the possibility of engineering pathways for production of high value carotenoids in methanotrophs.

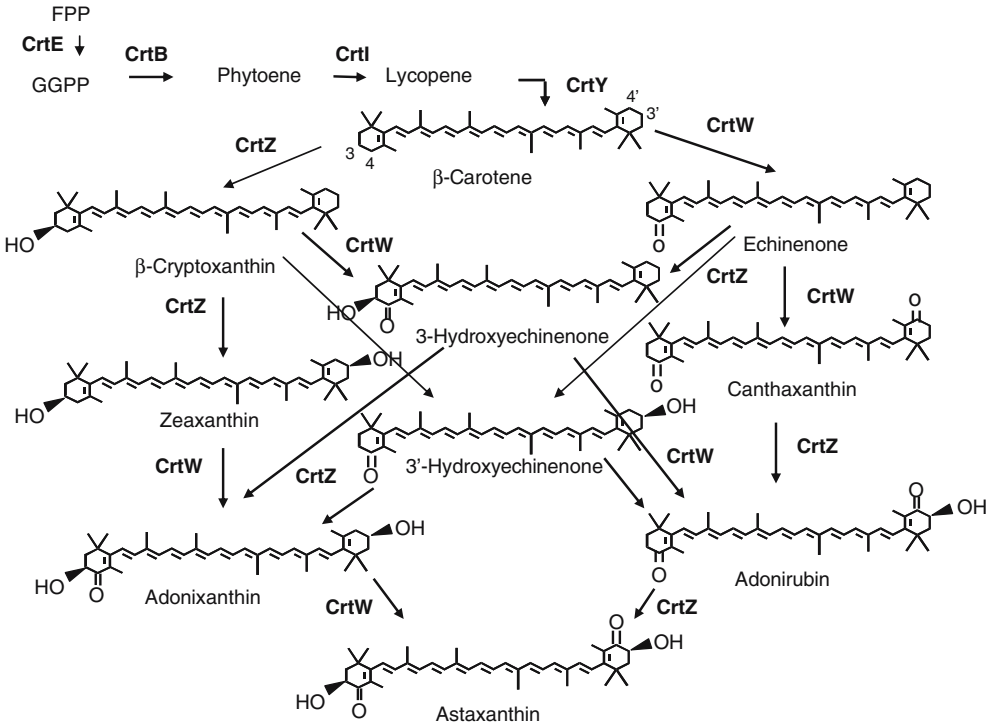

Fig. 1 Reactions of β-carotenoid ketolase and hydroxylase during the biosynthesis of astaxanthin

Materials and methods

Strains and growth conditions

Methylomonas sp. strain 16a (ATCC PTA-2402) is an environmental isolate. The wild type strain produces a pink C_{30} carotenoid pigment. The non-pigmented strain *Methylomonas* sp. MWM1200 (ATCC PTA-6887) was derived from the wild type by deletion of the promoter region that regulates the expression of the gene cluster containing *crtN*, *ald*, and *crtNb* that are involved in the C_{30} carotenoid biosynthesis.

Methylomonas sp. strain 16a and its derivatives were routinely grown in 150 or 500 ml serum bottles with PTFE backed butyl rubber stoppers (Wheaton Scientific; Wheaton, IL). The gas/liquid ratio was at least 8:1 in all experiments. The gas phase in the bottles contained 25% methane by volume with the balance as air. A minimal salts medium, BTZ, used was. Each liter of BTZ consisted of the following: 0.537 g NH_4Cl, 0.5 g KH_2PO_4, 0.5 g Na_2SO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 50 ml HEPES (1.0 M pH 7.0), and 10 ml trace mineral stock solution. Each liter of trace mineral stock solution consisted of 12.8 g nitriloacetic acid, 0.0254 g $CuCl_2{\cdot}2H_2O$, 0.3 g $FeCl_2{\cdot}4H_2O$, 0.1 g $MnCl_2{\cdot}4H_2O$, 0.312 g $CoCl_2{\cdot}6H_2O$, 0.10 g $ZnCl_2$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4{\cdot}2H_2O$, 0.184 g $NiCl_2{\cdot}6H_2O$. The pH of the solution was adjusted to pH 7.0 with NaOH. The cultures in bottles were grown at 30°C with constant shaking at 250 rpm. When grown on BTZ agar, plates were placed inside either a gas jar or gas box. The container was flushed with a stream of a gas mixture that consisted of 25% methane and 75% air by volume.

Construction of plasmids containing carotenoid biosynthetic genes

The construction of plasmid pDCQ333 was described previously [28]. This plasmid contained a complete set of *crt* genes for canthaxanthin production (Table 1). The β-carotenoid ketolase gene *crtW* from *Paracoccus* sp. N81106 (formerly classified as *Agrobacterium aurantiacum*) was codon-optimized for expression in *Methylomonas* sp. strain 16a (34; GenBank accession EF108323). The codon-optimized *crtW* shared 84%

Table 1 Bacterial strain and plasmids used in this study

Strains	Relevant characteristics	References
Strains		
E. coli Top 10	F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 galU	Invitrogen
Methylomonas sp. strain 16a	An environmental isolate using methane and methanol as the sole carbon source.	ATCC PTA-2402
MWM1200	Derirved from wild type 16a, non-pigmented due to a deletion in the promoter of *crtN ald crtNb* region	ATCC PTA-6887
Orihps333	Derived from MWM1200. The *crtWE idi crtYIB* cluster from pDCQ333 was integrated in the chromosome	ATCC PTA-7122
Ax6-5	Derived from Orihps333; *ald*::P_{hps}-*crtZ*	This study
Ax3-2	Derived from Orihps333; *ald*::P_{hps}-*crtWZ*	This study
Ax2-1	Derived from Orihps333; *ald*::P_{hps}-*crtZZ*	This study
Ax392	Derived from Ax6-5; *ccp*::*crtWZE idi crtYIB* The carotenoid gene cluster was from pDCQ392	This study
Plasmids		
pSUKSM	Km^r, an integration vector derived from pACYC [2]; containing *sacB* and *ori*T	This study
pSUKSMori	Derived from pSUKSM; contains a non-coding region for homologous recombination.	This study
pSUKSMorihps	Derived from pSUKSMori; P_{hps}	This study
pSUKSMald	Derived from pSUKSM; contains the *ald* region for homologous recombination	This study
pSUKSMaldhps	Derived from pSUKSMald; P_{hps}	This study
pBHR1	Broad-host range plasmid, Km^r, Cm^r	MoBiTec
pDCQ333	Derived from pBHR1, Km^r, contains *crtWE idi crtYIB.crtW* from *Paracoccus* sp. N81106; *crtE idi crtYIB* cluster from strain DC404	This study
pDCQ334	Derived from pDCQ333, Km^r, contains *crtWZE idi crtYIB* with *crtZ* from *Paracoccus* sp. N81106	This study
pDCQ343	Derived from pBHR1, Km^r, contains *crtWZEYIB*. *crtW* from DC18, *crtZ* from DC263, and *crtEYIB* from DC260	[28]
pDCQ392	Derived from pBHR1, Km^r, contains *crtWZE idi crtYIB* with both *crtW* and *crtZ* from DC263and *crtE idi crtYIB* from DC413	[28]

nucleotide identity with the native gene. In the resulting plasmid pDCQ333, the *crtWEidiYIB* cluster was flanked by EcoRI sites.

Plasmid pDCQ392 contained a complete set of genes for astaxanthin production (Table 1). The *crtW* gene from *Brevundimonas vesicularis* DC263 [28] was amplified using primers 5′-TCTAGAGCTAGCAAGG AGGAATAAACCATGCGGCAAGCG AACAG-GATG-3′ and 5′-ACTAGTCTAGCTGAACAAAC TCCACCAG-3′. The *crtZ* gene from strain DC263 was amplified using primers 5′-TCTAGAAAGGAG GAATAAACCATGTCCTGGCCGACGATGATC-3′ and 5′-ACTAGTCAGGCGCCGTTGCTGGATGA-3′. The XbaI-SpeI fragment containing the *crtW* gene and the XbaI-SpeI fragment containing the *crtZ* gene were cloned into the AvrII site upstream of the β-carotene synthesis genes on pDCQ380 [24]. The resulting construct was designated pDCQ392. The *crtW-ZEidiYIB* cluster was also flanked by two EcoRI sites.

Construction of canthaxanthin strain Orihps333 in *Methylomonas* sp. 16a

A non-coding region located at 13.6 kb down stream from the putative origin of replication of the genome was chosen to integrate the *crtWEidiYIB* gene cluster from pDCQ333 involved in canthaxanthin production. The putative origin of replication is next to the *dnaA* gene which encodes DnaA, a replication initiation factor. For integration through homologous recombination, this non-coding region was amplified by two primer pairs. Primers 5′-GCTCTAGAATTGGTA ATCTTCTGTTATTTATTG-3′ (forward) and 5′-CGGAATTCTCACGCCGTTTCGGCTGGTTGAA TG-3′ (reverse) were used to amplify the upstream region. An XbaI site was incorporated into the forward primer, while an EcoRI site was introduced in the reverse primer. To amplify the downstream region, primers 5′-CATTCAACCAGCCGAAACGGCGTG AGAATTCCTGATGTAGTTCAAACG-3′ (forward) and 5′-GAAGATCTTCGCCGTCCGCCATGCGCT AGCGGC-3′ (reverse) were used. The forward primer contained an EcoRI site while the reverse primer had a BglII site. After cloning both fragments into the XbaI and BglII sites of integration vector pSUSMK (Table 1), the resulting construct was designated pSUSMKori. In this construct, the EcoRI site in the middle of the non-coding region was available for cloning. The promoter for hexulose 6-diphosphate synthase gene (*hps*; GenBank accession number DQ631802) was amplified as a MfeI and EcoRI fragment with primers 5′-ATGACAATTGGTCGACGCGCTAAG GATTGGGGTGCGTCG-3′ (forward) and 5′-ATGA CAATTGCCTAGGGAATTCTGTACAGTGATGT GCTCCGAAAGTTT-3′ (reverse). The PCR fragment was cloned into the EcoRI site of pSUSMKori vector. The resulting construct was designated pSUMKorihps. In this construct, the EcoRI site downstream from the *hps* promoter was unique and was used to clone the canthaxanthin biosynthetic cluster *crtEidiYIB* from pDCQ333. This cluster was obtained by digesting the pDCQ333 vector with EcoRI. Cloning of the *crtEidiYIB* cluster into pSUSMKOriHps generated pSUSMKorihps333. This construct was transferred to *Methylomonas* sp. MWM1200 through conjugation and the *crt* gene cluster was integrated into the genome via homologous recombination.

Triparental mating was used to transfer DNA from *E. coli* donor to *Methylomonas* sp. strain 16a. Overnight cultures of the helper strain, donor strain and the non-pigmented *Methylomonas* MWM1200 were used at a ratio of 1:2:7 based on volume. The overnight cultures were collected by centrifugation and washed with BTZ medium before combining the cells together. The pellet containing all three strains was spotted onto a BTZ agar plate supplemented with 0.5% yeast extract. The mixture was incubated for 3 days at 30°C in a plastic box filled with 25% methane. The cultures were then spread onto BTZ agar plates supplemented with Kan (50 μg/ml). Single colonies were than streaked onto another BTZ plate. This process was repeated to purify the *Methylomonas* exconjugants away from the *E. coli* cells. The double-cross over strains were enriched by repeated sub-culturing in liquid medium without Kan and plated on BTZ plates supplemented with sucrose (5%, freshly prepared). The individual colonies that were sensitive to Kan were selected and further characterized.

Construction of *Methylomonas* sp. strains Ax6-5, Ax3-2 and Ax2-3

The biosynthesis of astaxanthin requires both the CrtW ketolase and CrtZ hydroxylase. The DNA region containing *crtN, ald*, and c*rtNb* genes was chosen for the integration of different combinations of *crtZ* and *crtW* genes for astaxanthin biosynthesis. This DNA region was involved in the native pigment production [5] and was shown to be non-essential for cell growth (unpublished observation). The 5′ DNA region used for homologous recombination was amplified with primer set 5′-TTCTAGAAAAGCCAAAGCCTGAGT ATGACGA-3′ (forward) and 5′-CGCAATTGAATTC GTTTAAACAGTACTTCATTAGTCATCCCGTG TCCAAGAA-3′ (reverse). The 3′ DNA region was amplified with another primer set 5′-T TCTTGGAC ACGGGATGACTAATGAAGTACTGTTTAAACG

AATTCAATTGACTCAAATGACAACCAACGCG TGATC-3′ (forward), and 5′-GAAGATCTCCCG GACAGCGTCACCATCGGCATG-3′ (reverse). The subsequent amplification of the entire region was carried out with both fragments as the template using the forward primer of the upstream region and the reverse primer of the downstream region. The PCR product was digested with XbaI and BglII and cloned into the integration vector pSUSMK, resulting in the construction of pSUSMKald. This plasmid was further modified by introduction of the *hps* promoter. The *hps* promoter was obtained as an EcoRI fragment with the primer set 5′-ATGAGAATTCGTCGACGCGCTAAGGATTG GGGTGCGTCG-3′ (forward) and 5′-pSUSM Kaldhps. The *crtZ* was amplified as a BsrGI and AvrII fragment from pDCQ334 plasmid (Table 1). The two primers used were 5′-GGCCATGCTGTACATCTAGAAA GGAGGAATAAACCATGACCA (forward) and 5′-C GCGTACGCCTAGGTTAGGTGCGTTCTTGGGC TTCGGCA (reverse). Cloning of the *crtZ* gene into the BsrGI and AvrII site generated plasmid pSUSMKaldhps-crtZ. In this construct, the *crtZ* gene was under the control of the *hps* promoter. The plasmid pSUSMKaldhps-crtZ was introduced into the canthaxanthin producing strain Orihps333 through conjugation as described above. The integration strain obtained was designated as *Methylomonas* sp. strain Ax6-5 (Table 1).

To construct strain Ax3-2, the *crtW* and *crtZ* genes were amplified as an MfeI and AvrII fragment with pDCQ334 as the template DNA. The primers used were 5′-GGCCATGCCAATTGACTAGAAAGGAG GAATAAACCATGACCGTCGATCACGACGCA (forward) and CGCGTACGCCTAGGTCAGGCGCC GTTGCTGGATGAGCCGCGT-3′ (reverse). The DNA fragment obtained was cloned into the corresponding sites in plasmid pSUSMKaldhps, generating pSUSMKaldhps-crtWZ. This plasmid was introduced into strain 16a via conjugation, leading to the construction of Ax3-2.

In strain Ax2-3, two copies of *crtZ* gene under the control of a single *hps* promoter were integrated into the chromosome of Orihps333. The first copy of the *crtZ* gene was derived from pDCQ334 and it was amplified with primer set 5′-GACGACGACAAGAT TGTACATCTAGAAAGGAGGAATAAACCATG ACCAACTTCCTGA-3′ (forward) and 5′-CGCGGG CGGCCGTTCTTAGGTGCGTTCTTGGGCTTCGG C-3′ (reverse). The second copy of the *crtZ* gene was derived from pDCQ343 and it was amplified with primers 5′-CGGCCGCCCGCGTTGACTAGAAAG GAGGAATAAACCATGTCCTGGCCGACGATG-3′ (forward) and 5′-GAGGAGAAGCCCGGTCCCT AGGTCAGGCGCCG TTGCTGGATGAGCC-3′ (reverse). These two fragments were assembled together with ligation independent cloning method based on the pET30-EK/LIC vector kit (Novagen). The *crtZZ* construct was then cloned as a BsrG I and Avr II fragment into pSUSMKaldhps. Integration of this construct in strain Orihps333 led to the construction of strain Ax2-3.

Construction of *Methylomonas* sp. strain Ax392

In order to further improve the titer and selectivity for astaxanthin production, another copy of the entire astaxanthin biosynthetic gene cluster *crtWZEidiYIB* from pDCQ392 (Table 1) was integrated into strain Ax6-5. The location for integration was the DNA region *ccp* encoding the cytochrome *c* peroxidase. This region was previously identified by random transposition and was shown to support a high level of expression of *crt* genes (Sharpe et al., unpublished observation). To construct the integration vector, the upstream region of *ccp* was amplified with primer set 5′-GCATG CTAGCCGACGGCTGATCGCATGCTGGCTTATC A-3′ (forward) and 5′-GCATACTAGTTACGCGC GCCAACACCGTTGACATGAAA-3′ (reverse). The downstream region was amplified with the primer set 5′-GCATGAATTCGGGTAGAGCCGCGTAA TGTGCCGACC-3′ (forward) and 5′-GCTAAGA TCTGATGATCGCCTGTCTATCCTCGGGAGC A-3′ (reverse). The upstream region was cloned as an NheI and SpecI fragment into the XbaI site of the integration vector pSUSMK. The downstream arm was cloned as a EcoRI and BglII fragment, generating the construct pSUSMKccp. The gene cluster *crtWZEidiYIB* as an EcoRI fragment from pDCQ392 was cloned in the EcoRI site of pSUSMKccp. The final construct was introduced into strain Ax6-5 via conjugation as described above. The resulting strain was designated Ax392. In this strain, the expression of *crtWZEidiYIB* gene cluster was under the control of the native promoter for the *ccp* gene.

Batch fermentation study of *Methylomonas* sp. strain Ax392

A 2 l Braun Biostat B bioreactor (Sarotorius, Allentown, PA) with a working volume of 1.6 l was used for this study. The medium used for the fermentation was essentially the same as BTZ, except that the amount of nitrogen source and trace minerals was doubled. The starting culture was grown for 48 h in 500 ml Wheaton serum bottles with 50 ml of unmodified BTZ medium. After inoculating 250 ml of the starting culture (15.6%

of the final volume), the initial cell density in the bioreactor was 0.32 O.D. as measured by optical density at 600 nm. During the run, the pH was maintained constantly at 7.0 ± 0.05 with 5 M NaOH. Methane, nitrogen and oxygen were delivered to the medium through the use of individual Brooks MFx series mass flow controllers (Brooks Instruments, Hatfield, PA). Prior to entering the fermentor the gas flows were mixed and delivered at a total gas flow rate of 875 ml/min. The gas mixture consisted of 26.3% methane, 53.1% nitrogen, and 20.6% oxygen by volume. The back pressure of the reactor was 0.25 psi. The dissolved oxygen was maintained at 20% of air saturation during the entire run through set point control using the stirrer rate as the manipulated variable. The stir rate ranged from 297 to 648 rpm, depending on the oxygen demand of the culture. The temperature of the culture was maintained at 30°C.

Analysis of carotenoids

Extraction of carotenoids in *Methylomonas* sp. 16a was routinely carried out with a 10 ml cell culture as previously described [34]. The extracted samples were analyzed by high performance liquid chromatography (Beckman Coulter, Inc., Fullerton, CA) using the same column and eluent conditions as described. The individual carotenoids were quantified based on authentic standards (CaroteNature GmBH, Lupsingen, Switzerland). When necessary the identity of different carotenoids was confirmed by spectral comparison with known standards and mass spectrometry (Agilent Technologies, Inc., Wilmington, DE).

Results

Identification of the native *hps* promoter for constitutive gene expression

Constitutive promoters that are active during cell growth can be used to express genes involved in carotenoid biosynthesis. The obvious candidates are those that control the expression of genes involved in methane oxidation, methanol utilization and formaldehyde assimilation. Our preliminary DNA microarray experiment found that the *pmoC*, *moxF*, and *hps* genes were some of the highly expressed regions in the genome. These three regions appear to be the first genes in their respective operons. The *pmoC gene* encodes the PmoC subunit of the particulate methane monooxygenase [10], while the *moxF* encodes the alpha subunit of the methanol dehydrogenase [26]. The *hps* gene encodes the 3-hexulose-6-phosphate synthase which catalyzes the first step in the RuMP pathway for formaldehyde assimilation [12, 32]. The mRNA levels of these genes were further investigated by RT-PCR with 16S ribosomal RNA as the internal control (Table 2). Based on the threshold cycle (C_T) values, the mRNA levels for all three genes were similar in their abundance. In this study, the promoter region of the *hps* gene was chosen to express genes involved in carotenoid biosynthesis.

Expression of the gene cluster involved in canthaxanthin in the chromosome of *Methylomonas* sp. strain 16a

The strategy to engineer *Methylomonas* sp. strain 16a was to express an appropriate biosynthetic gene cluster in the chromosome instead of on a plasmid. This would circumvent issues of using antibiotic resistance as a selection pressure, plasmid burden, and segregation stability in long-term cultivation. In order to obtain a stable strain, non-essential regions of the chromosome were searched for integration. A non-coding region near the putative origin of replication was targeted. A previously constructed canthaxanthin gene cluster from pDCQ333 [28] was placed under the control the *hps* promoter in the integration vector. Integration of this gene cluster in the non-coding region in the non-pigmented *Methylomonas* strain MWM1200 led to the construction of strain Orihps333. The colonies of Orihps333 were orange in color, indicating that the *crt* genes in the cluster were expressed.

Initially, strain Orihps333 was characterized for its canthaxanthin production by growing the culture in 40 ml of BTZ medium in 500 ml bottles with methane as the carbon source. Under these growth conditions, canthaxanthin produced was about 65% of the total

Table 2 RT-PCR quantification of mRNA levels of different genes in *Methylomonas* sp. 16a

Genes	Function	ΔC_T values ($C_{T,16S} - C_{T,X}$)
moxF	Methanol dehydrogenase (large subunit)	−9.26 ± 0.56
pmoC	Particulate methane monooxygenase (subunit C)	−8.42 ± 0.67
hps	Hexulose 6-phosphate synthase	−9.02 ± 0.49
ccp	Cytochrome *c* peroxidase	−12.72 ± 0.85

The ΔC_T values were used to reflect the relative abundance of mRNA of specific genes normalized to the 16S mRNA levels. $C_{T,16S}$ was the threshold cycle for 16S mRNA, while the $C_{T,X}$ was the threshold cycle for a specific target gene. The RT-PCR was carried out with the ABI 7,900 SDS instrument (Applied Biosystems, Foster City, CA, USA)

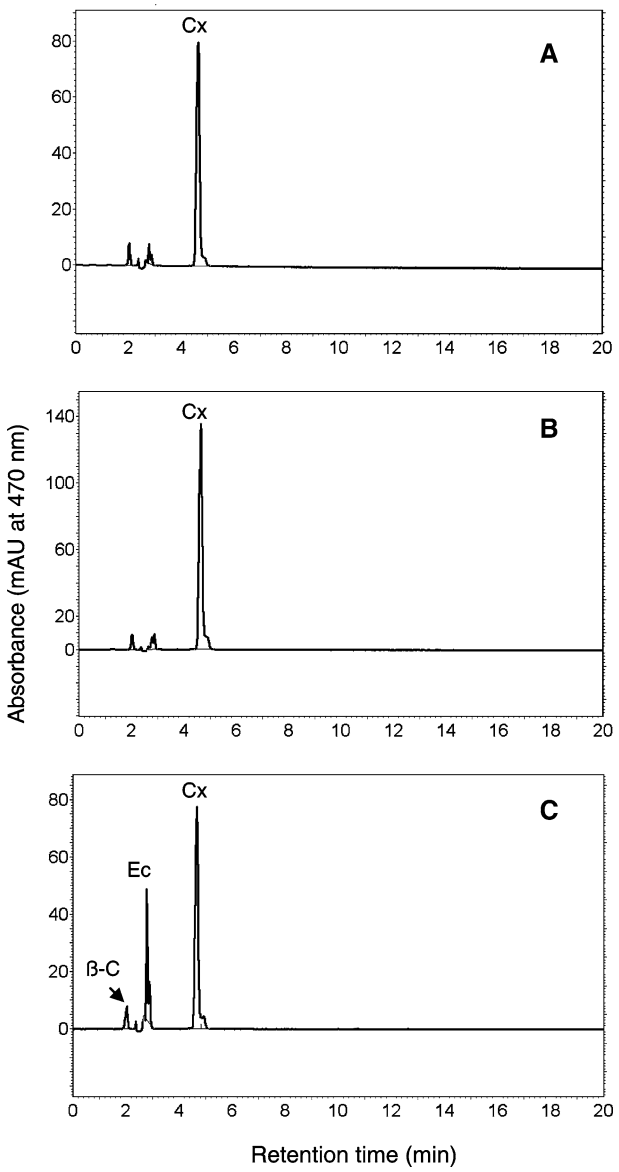

Fig. 2 HPLC analysis of the carotenoid profiles for the canthaxanthin-producing strain Orihps333. The strain was grown using methane as the carbon source and ammonia as the nitrogen source. Experiment was carried out in 10 ml (**a**), 20 ml (**b**), and 40 ml (**c**) of BTZ medium in 500 ml serum bottles. Extraction was carried out with cells grown for 24 h. Commercial authentic carotenoids were used as standards. Identities of different carotenoid intermediates were also confirmed by optical spectrums and mass spectrometry [34] Cx Canthaxanthin, Ec echinenone, β-C β -carotene

carotenoid present in the culture (Fig. 2). The other carotenoid intermediates were echinenone and β-carotene. It is likely that the availability of oxygen or oxygen transfer efficiency in bottles with 40 ml medium was limited. These growth conditions had a negative impact on the canthanxanthin selectivity. When strain Orihps333 was grown in 10 and 20 ml of growth medium, the canthaxanthin selectivity was increased to 90% and the intermediates echinenone and β-carotene were only present as minor components. The above result suggests that culture conditions in addition to genetic construction play a role in optimizing canthaxanthin selectivity. The titer for canthaxanthin in bottle experiments was about 0.8 mg g^{-1} based on dry cell weight (DCW). Strain Orihps333 had a maximum growth rate of 0.28 h^{-1} and the canthaxanthin-producing phenotype appeared to be stable after repetitive sub-culturing.

Production of astaxanthin by expressing different combinations of *crtW* and *crtZ* genes in strain Orihps333

As shown in Fig. 1, modifications of the two β-ionone rings by the CrtW ketolase and CrtZ hydoxylase can lead to the production of different intermediates during the biosynthesis of astaxanthin. Three strains were constructed to investigate the intermediate profiles when different combinations of *crtW* and *crtZ* genes under the control of the *hps* promoter were integrated in the chromosome in the Orihps33 strain background. The chromosomal location used for integration was the *ald* region that encodes the aldehyde dehydrogenase. The *ald* gene is located in the same operon as the *crtN* and *crtNb* genes that are involved in the native pigment production and is not essential for cell growth [5]. The first strain constructed, Ax6-5, contained a single copy of the *crtZ* gene integrated in the *ald* region. The second strain Ax2-1, on the other hand, contained two copies of the *crtZ* genes in tandem in this location. The third strain, Ax3-2, had a *crtW* and a *crtZ* gene as an operon integrated the *ald* region. This strain contained two copies of the *crtW* gene including the one associated with the canthaxanthin biosynthetic cluster. The carotenoid product profiles of these three strains are shown in Fig. 3. Strain Ax6-5, which had a single copy of the *crtZ* and *crtW*, produced both astaxanthin and the mono-ketolated intermediate adonixanthin. The astaxanthin consisted of 64% of the total carotenoid, while adonixanthin was 32%. In strain Ax2-1, which had two copies of *crtZ* and a copy of *crtW*, the amount of adonixanthin increased to 60%. This indicated that CrtW ketolase activity was not adequate enough to complete the ketolation reaction in both strains. In contrast, strain Ax3-2, which contained two copies of *crtW* and one copy of *crtZ*, showed an increase in the amount of canthaxanthin and adonirubin. Both canthaxanthin and adonirubin contain two keto-groups and require hydroxylation to be converted to astaxanthin. Although none of these strains had satisfactory selectivity for astaxanthin production, their carotenoid intermediate profiles

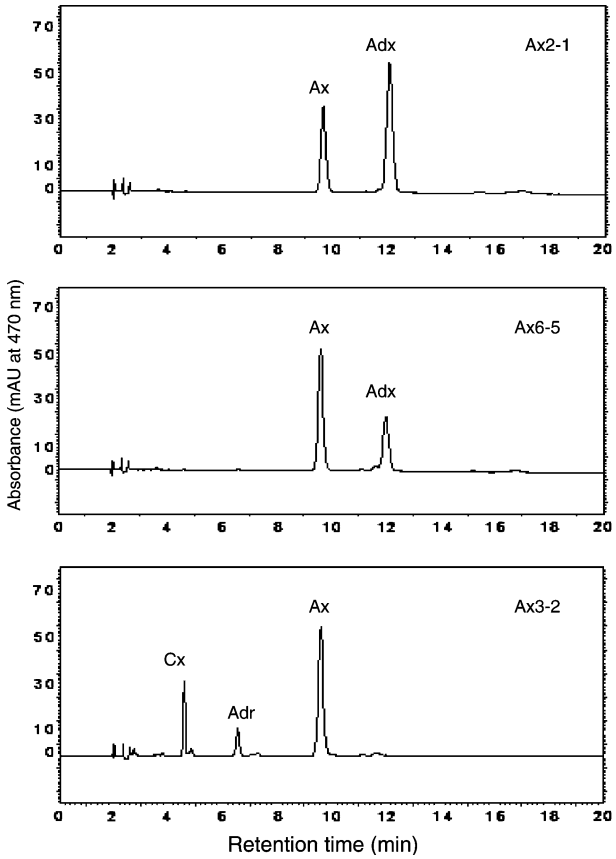

Fig. 3 HPLC analysis of carotenoid profiles of different astaxanthin producing strains when grown in 500 ml serum bottles with 10 ml BTZ medium Ax astaxanthin, Cx canthaxanthin, Adx adonixanthin, Adr adonirubin

reflected the relative activity levels of the CrtZ hydroxylase and CrtW ketolase.

Increasing astaxanthin conversion by expressing a second copy of astaxanthin gene cluster

Strain Ax6-5 had the highest selectivity for astaxanthin production among the three astaxanthin-producing strains and was used for further optimization. This was carried out by the integration of another complete gene cluster for astaxanthin biosynthesis. The gene cluster from pDCQ392 (Table 1) was used for integration since these *crt* genes have limited similarity to those used for the construction of Orihps333 and strain Ax6-5 [24, 28]. The *crtW* and *crtZ* genes in this cluster were organized as an operon along with *crtE*, *idi*, *crtY*, *crtI* and *crtB*. The chromosomal region for integration was *ccp* that encodes the cytochrome *c* peroxidase. This location was identified to support a high level of expression of *crt* genes by a random transposition method (unpublished observation). Integration of this new astaxanthin biosynthetic gene cluster in the *ccp* region resulted in the construction of strain Ax392. In this strain, the expression of the *crt* genes was presumably under the control of the same native promoter as the *ccp* gene. When this strain was grown in 10 ml BTZ medium in a 500 ml serum bottle, the astaxanthin selectivity was significantly increased as compared to strain Ax6-5. Strain Ax392 produced astaxanthin as the predominant product (Fig. 4). In fact, up to 95% of the total carotenoid in the culture was astaxanthin. Among the different forms of astaxanthin isomers, the all *E*-isomer was 89%. This geometric isomer is the form of astaxanthin that salmonid fish can easily uptake [23]. The titer of astaxanthin in Ax392 strain when grown in 10 ml medium was 1.5 ± 0.5 mg g^{-1} based on DCW. This result indicated that the addition of another copy of the astaxanthin biosynthetic cluster improved both astaxanthin selectivity and titer.

Astaxanthin selectivity of strain Ax392 under batch fermentation conditions

To investigate whether a high astaxanthin selectivity can be achieved under batch fermentation conditions, a bioreactor was set up to further characterize strain Ax392. Unlike the closed bottle experiment, both methane and oxygen were directly delivered and mixed into the growth medium in the reactor. By keeping the dissolved oxygen constant, consistent culture conditions with respect to dissolved gas concentrations and mass trasnfer characteristics could be maintained. Under these growth conditions, the initial growth rate of the strain was 0.26 h^{-1}. During the growth phase, the selectivity of astaxanthin was in the range of 40–60% (Fig. 5). The other significant carotenoid intermediates were canthaxanthin and adonirubin, both of which are ketolated products. When the growth rate started to slow down due to ammonia limitation, the astaxanthin selectivity increased to 85%, while the percentage of

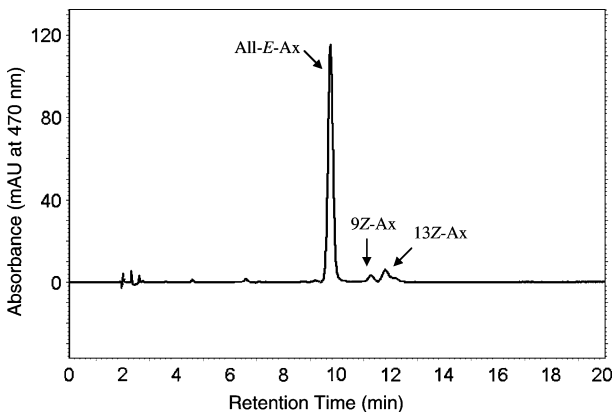

Fig. 4 Astaxanthin production of Ax392 when grown in 500 ml serum bottles with 10 ml BTZ medium Ax, astaxanthin

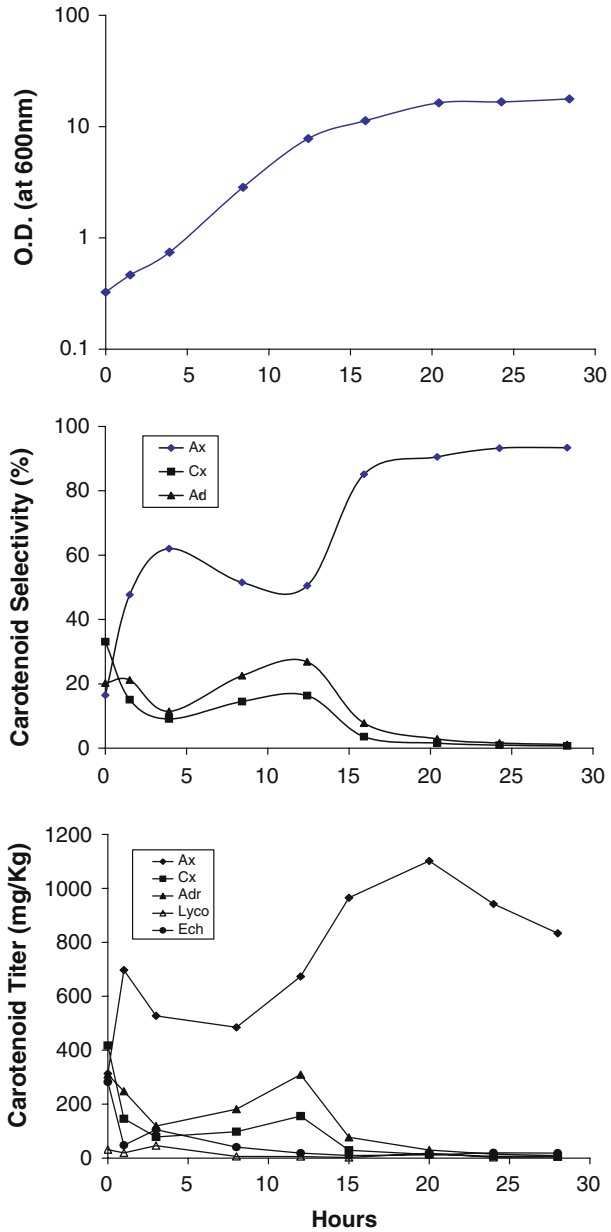

Fig. 5 Selectivity of astaxanthin for strain Ax392 under batch fermentation conditions. *Ax* astaxanthin, *Cx* canthaxanthin, *Adr* adonirubin Lyco, lycopene

canthaxanthin and adonirubin decreased. A selectivity of 90% or higher was achieved after a few hours at the stationary phase. The result from the batch experiment indicated that the efficiency of astaxanthin conversion in strain Ax392 was influenced by the growth stage of the culture and the environmental conditions in the fermenter. The decrease in growth rate and changing nutrient conditions at the end of the fermentation run also play a role in improving astaxanthin selectivity in this strain. A further improvement in titer, productivity and fermentation conditions for this strain is needed to improve the economy of the production process.

Discussion

Chromosomal integration is one method to strain construction that circumvents the issues of segregation stability and selection pressure associated with plasmid-based gene expression systems. Since methanotrophic fermentations are largely governed by mass transfer limitation and explosive concerns. Economic goals and safety issues require complete conversion of oxygen and methane in a process that is stable, reliable, and easily controlled. For these issues and additional productivity considerations, continuous fermentation is one of the likely the processing modes to be used with this type of system. Long-term continuous cultivation provides additional challenges to working with engineered systems in general and plasmid-based gene expression systems in particular. For these reasons, integration into the chromosome was the approach that we used to construct the pathway involved in carotenoid biosynthesis in *Methylomonas* sp. 16a.

Two of the regions used for the initial integrations of *crt* genes were a non-coding region near the putative origin of replication and the *ald* region involved in the native pigment production. The native *hps* promoter was used in these two locations for gene expression. The third region used for integration and expression of *crt* genes was *ccp* that encodes the cytochrome *c* peroxidase. Thus, the construction of astaxnthin-producing strain Ax392 was a result of combining the genome information and experimental results from various approaches. Despite the fact that multiple genes and clusters were integrated in the chromosome, strain Ax392 appeared to have a very stable phenotype as evidenced by its survival in long continuous fermentation campaigns (unpublished observation). These results suggest that these locations and the *hps* promoter are suitable for integration and expression of foreign genes for the purpose of pathway engineering in this organism.

During the construction of astaxanthin-producing strains in *Methylomonas* sp. strain 16a, we encountered the challenge of obtaining a desirable selectivity due to the incomplete conversion of different carotenoid intermediates. A lack of adequate activities of CrtZ hydroxylase and CrtW ketolase was likely the explanation for some of the initial strains, though contributions by growth rate and environmental influences can not be ruled out based on the findings of this report. Both these two enzymes have different substrate specificities for different intermediates and prefer substrates without prior substitutions to the β-ionone rings [8, 9]. The other explanation could be related to the physiology of

Methylomonas sp. strain 16a, growth conditions, and the additional requirements associated with using methane as the carbon source. A proper balance between the growth on methane and astaxanthin biosynthesis is required to achieve optimal astaxanthin conversion.

We have recently shown that the efficiency of astaxanthin production in *E. coli* can be improved by mutagenizing the *crtW* gene [34]. The improved mutants identified were not available during the construction of astaxanthin-producing strains in *Methylomonas* sp. strain 16a. As a result, these mutants were tested in this organism. It is observed that the expression of the *crtW* gene from *Brevundimonas* sp. SD212 in *E. coli* leads to an efficient conversion of adonixanthin to astaxanthin [6], suggesting sources of the *crtW* gene confer different selectivity. We have also recently shown that expression of hemoglobin genes can improve the astaxanthin selectivity when engineered *Methylomonas* strains were grown in small wells [29]. Whether the improvement can be observed under fermentation conditions remains to be determined. In the experiment described here, a high astaxanthin selectivity was achieved by expressing different combination of *crtW* and *crtZ* genes. All the above results indicate that multiple approaches can be employed to increase the astaxanthin selectivity. Although *E. coli* carrying *Brevundimonas* sp. SD212 *crtZ* showed the highest astaxanthin production efficiency among the *crtZ* genes examined [7], the approach to improve the CrtZ hydoxylase by protein engineering remain to be explored. The discovery of a single enzyme with both hydroxylase and ketolase activities in *X. dendrorhous* [2, 22] provides another tool for pathway engineering for astaxanthin biosynthesis.

Engineering carotenoid biosynthetic pathways have been investigated in various non-carotenogenic microbes, including *E. coli* [1, 35] and Yeast [21, 25, 33]. All these systems require glucose or other multicarbon substrates. Methane can be an attractive carbon source to be used to generate high value products in places where there is abundance of natural gas or where availability of low cost sugar is not an option. Successful construction of the astaxanthin biosynthetic pathway in *Methylomonas* sp. strain 16a demonstrates the possibility of engineering methanotrophs and the use of methane as the feedstock for bioprocessing. A fish trial study with salmon has been completed with the biomass generated from strain Ax392. The result showed that the fish can be colored with the astaxanthin produced in this strain, further substantiating the potential of using methanotrophs for commercial applications.

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