Chapter 10

Construction of Carotenoid Biosynthetic Pathways Through Chromosomal Integration in Methane-Utilizing Bacterium *Methylomonas* sp. Strain 16a

Rick W. Ye and Kristen Kelly

Abstract

Methylomonas sp. strain 16a is an obligate methanotrophic bacterium that uses methane or methanol as the sole energy and carbon source. In order to engineer a stable strain to produce carotenoids, integration of genes or gene clusters in various nonessential locations in the chromosome is used. Construction of a canthaxanthin-producing strain involves the integration of canthaxanthin biosynthetic genes including the *crtW* gene for the β-carotenoid ketolase. Addition of the *crtZ* gene that encodes the β-carotenoid hydroxylase in this strain leads to the production of astaxanthin. Further increase in titer and yield for astaxanthin is obtained by integration of another set of astaxanthin biosynthetic gene cluster in a separate location of the chromosome.

Key words: Methane, Methanotroph, Pathway engineering, Chromosomal integration, Conjugation, Homologous recombination, Carotenoids, Canthaxanthin, Astaxanthin

1. Introduction

Obligate methanotrophs are those bacteria that can only grow on methane or methanol. However, examples of engineering this group of organisms to produce commercial products remain scarce. It can be difficult to introduce a complex pathway into methanotrophs since these organisms are not well studied and the genetic systems are less developed as compared to those for *E. coli* and other industrial organisms. In addition, delivering methane gas to the organism as carbon source is not as convenient as using sugars as substrates. Despite these drawbacks, methanotrophs have been considered as production hosts due to the natural abundance of methane. Furthermore, methane can be generated from biomass or biowaste using microbial processes.

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Canthaxanthin and astaxanthin are carotenoids that have been widely used commercially (1). Biosynthesis of carotenoids is derived from the isoprenoid pathway. β-carotene can be made from farnesyl pyrophosphate by expression of *crtE*, *crtY*, *crtI*, and *crtB* genes (2–4). The two β-ionone rings in β-carotene are modified by ketolases and hydroxylases to produce canthaxanthin and astaxanthin. In some bacterial systems, the β-carotene hydroxylase CrtZ introduces the hydroxyl groups on the β-ionone rings, while the ß-carotene ketolase CrtW catalyzes the reaction of adding two keto-groups (5, 6). Only the CrtW ketolase is necessary for the production of canthaxanthin, while a combination of both CrtZ hydroxylase and CrtW ketolase is required for the biosynthesis of astaxanthin. Based on color intensity of bacterial colonies, improvement of canthaxanthin and astaxanthin production can be achieved by screening mutation libraries of ketolase CrtW (7, 8).

To demonstrate the feasibility and the commercial potential of engineering complex pathways in methanotrophs, here we describe a procedure for the construction of canthaxanthin- and astaxanthin-producing strains in *Methylmonas* sp. strain 16a. To maintain strain stability, which is required for large-scale fermentation process, construction of these strains is achieved through integration of the carotenoid pathway genes in the chromosome and manipulation of the expression level with appropriate promoters and gene copy numbers.

2. Materials

In addition to the routine techniques to handle microorganisms and molecular biological materials, care must be taken to manipulate methane gas due to its explosive nature.

2.1. Strains and Plasmids

Additional information about strains, plasmids, and gene clusters can be found in Table 1.

1. *Methylomonas* sp. 16a ATCC PTA-2402 (ATCC, Manassas, VA, USA).
2. *Methylomonas* MWM1200 ATCC PTA-6887 (ATCC, Manassas, VA, USA) (see Note 1).
3. *E. coli* pRK2013 ATCC 37159 (ATCC, Manassas, VA, USA).
4. pSUKSM (9) (see Note 2).
5. pSUKSMori (9).
6. pSUKSMorihps (9).
7. pSUKSMald (9).
8. pSUKSMaldhps (9).

Table 1
Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	References
Strains		
Methylomonas sp. 16a	WT	ATCC PTA-2402
MWM1200	Non-pigmented *Methylomonas*	ATCC PTA-6887
E. coli with pRK2013	Helper strain for conjugation	ATCC 37159
Plasmids		
pSUKSM	Km^r, an integration vector derived from pACYC containing *sacB* and *oriT*	(9, 13)
pSUKSMori	Derived from pSUKSM; contains a noncoding region for homologous recombination	(9)
pSUKSMorihps	Derived from pSUKSMori; P_{hps}	(9)
pSUKSMald	Derived from pSUKSM; contains the *ald* region for homologous recombination	(9)
pSUKSMaldhps	Derived from pSUKSMald; P_{hps}	(9)
pDCQ333	Derived from pBHR1, Km^r; contains *crtWE idi crtYIB*. *crtW* from *Paracoccus* sp. N81106 (GenBank: D58420); *crtE idi crtYIB* cluster from strain DC404 (GenBank: DQ090834)	(9)
pDCQ334	Derived from pDCQ333, Km^r; contains *crtWZE idi crtYIB* with *crtZ* from *Paracoccus* sp. N81106 (GenBank: D58420)	(10)
pDCQ392	Derived from pBHR1, Km^r; contains *crtWZE idi crtYIB* with both *crtW* and *crtZ* from DC263 (GenBank: DQ309446) and *crtE idi crtYIB* from DC413 (GenBank: DQ090836)	(10)

9. pDCQ333 (10).
10. pDCQ334 (9).
11. pDCQ392 (9).

2.2. Growth Medium for Methylomonas and E. coli

1. Luria–Bertani (LB): Add 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl into 1 L of deionized water. Autoclave at 121°C for 15 min.
2. LB-agar: LB and 15 g/L agar. Autoclave at 121°C for 15 min.
3. Kanamycin (Kan) stock solution (50 mg/mL).
4. BTZ medium: 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. Bring with distilled water to 1 L. Autoclave at 121°C for 15 min.
5. BTZ-agar: BTZ and 15 g/L agar. Autoclave at 121°C for 15 min.

6. Trace mineral stock solution: 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 $Na_2MoO_4 \cdot 2H_2O$, and 0.184 g $NiCl_2 \cdot 6H_2O$. Bring with distilled water to 1 L. Adjust the pH of the solution to pH 7.0 with NaOH solution. Autoclave at 121°C for 15 min.

2.3. Growth Apparatus for Methylomonas

1. Serum bottles (150 or 500 mL) sealed with PTFE backed butyl rubber stoppers (Wheaton Scientific; Wheaton, IL, USA) (see Note 3).
2. Syringe (see Note 3).
3. Gas jar or gas box (see Note 4).

2.4. Primer List

1. A: 5′-GCTCTAGAATTGGTAATCTTCTGTTATTTATTG-3′.
2. B: 5′-CGGAATTCTCACGCCGTTTCGGCTGGTTGAATG-3′.
3. C: 5′-CATTCAACCAGCCGAAACGGCGTGAGAATTCCT GATGTAGTTCAAACG-3′.
4. D: 5′-GAAGATCTTCGCCGTCCGCC ATGCGCTAGCGGC-3′.
5. E: 5′-ATGACAATTGGTCGACGCGCTAAGGATTGGGGT GCGTCG-3′.
6. F: 5′-ATGACAATTGCCTAGGGAATTCTGTACAGTGATG TGCTCCGAAAGTTT-3′.
7. G: 5′-TTCTAGAAAAGCCAAAGCCTGAGTATGACGA-3′.
8. H: 5′-CGCAATTGAATTCGTTTAAACAGTACTTCATTAG TCATCCCGTGTCCAAGAA-3′.
9. I: 5′-TTCTTGGACACGGGATGACTAATGAAGTACTGTT TAAACGAATTCAATTGA CTCAAATGACAACCAACGCGTGATC-3′.
10. J: 5′-GAAGATCTCCCGGA CAGCGTCACCATCGGCATG-3′.
11. K: 5′-GGCCATGCTGTACATCTAGAAAGGAGGAATAAAC CATGACCA-3′.
12. L: 5′-CGCGTACGCCTAGGTTAGGTGCGTTCTTGGGCT TCGGCA-3′.
13. M:5′-GCATGCTAGCCGACGGCTGATCGCATGCTGGCT TATCA-3′.
14. N: 5′-GCATACTAGTTACGCGCGCCAACACCGTTGACA TGAAA-3′.
15. O: 5′-GCATGAATTCGGGTAGAGCCGCGTAATGTGCCG ACC-3′.
16. P:5′-GCTAAGATCTGATGATCGCCTGTCTATCCTCGGG AGCA-3′.

3. Methods

For industrial applications where a large volume of product is made, it is desirable to have a stable strain. One approach to construct such strain is through the integration of various carotenoid genes or gene clusters in different locations of the chromosome. These locations have to be nonessential, but allow an adequate level of expression of the integrated genes.

Integration of recombinant genes into *Methylomonas* is obtained using an integration vector which cannot replicate in this organism. Classical triparental mating is used to transfer DNA from *E. coli* donor to *Methylomonas*. *Methylomonas* sp. Strain 16a is sensitive to Kan and thus the Kan-resistant gene can be used as the selection marker. After conjugation, the first event of homologous recombination is selected based on resistance to Kan due to the integration of the vector by single crossover. Addition of sucrose in the growth medium selects for the second recombination event which results in the excision of the plasmid from the chromosome (11, 12).

3.1. Cloning of the Canthaxanthin Gene Cluster in the Integration Vector

1. The first step to construct the integration vector is to select a DNA region for homologous recombination. In this case, a noncoding region located 13.6 kb downstream from the putative origin of replication of the genome is chosen to integrate the canthaxanthin cluster through homologous recombination (see Note 5).
2. Amplify the 1.1-kb homologous upstream region with primers A and B. An XbaI site is incorporated into the primer A, while an EcoRI site is introduced in the primer B.
3. Amplify the 1.3-kb downstream region with primers C and D. The primer C contains an EcoRI site while primer D has a BglII site.
4. Clone the two fragments into the XbaI and BglII sites of the integration vector pSUSMK. The resulting plasmid is pSUSMKori (Fig. 1), which has a single EcoRI site in the middle of the noncoding region available for cloning of a promoter and the carotenoid gene cluster for integration.
5. Amplify the *hps* promoter (see Note 6) as an MfeI and EcoRI fragment with primers E and F.
6. Clone the PCR fragment into the EcoRI site of pSUSMKori vector. The resulting plasmid is pSUMKorihps, which has a single EcoRI site downstream from the *hps* promoter.
7. Digest pDCQ333 with EcoRI to obtain a fragment containing the canthaxanthin biosynthetic cluster *crtEidiϒIB* (Table 1).

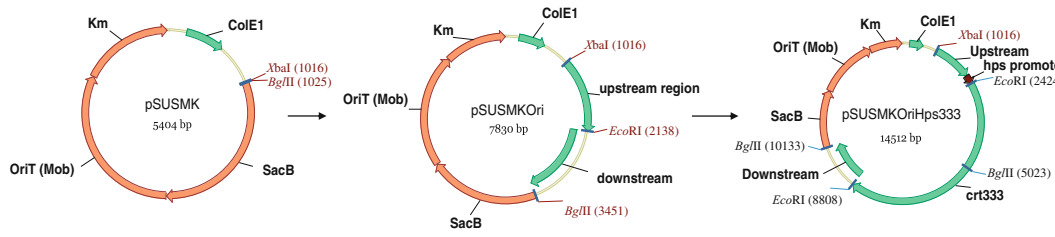

Fig. 1. Construction of integration vector for expression of carotenoid genes in the chromosome. The first step is the cloning of the genomic regions targeted for integration. The second step is the introduction of the *crt* genes between these two regions.

8. Clone this fragment into the EcoRI site of the integration vector pSUMKorihps. The resulting plasmid with proper orientation to the *hps* promoter is pSUSMKorihps333 (Fig. 1).

3.2. Integration of the Canthaxanthin Pathway Through Conjugation and Homologous Recombination

The procedure is outlined in Fig. 2.

1. Start overnight cultures of the helper (*E. coli* strain with pRK2013) and the donor (*E. coli* strain with the integration plasmid pSUSMKorihps333) in LB medium supplemented with Kan (50 μg/mL), and incubate the culture at 37°C.
2. Start a culture of the non-pigmented MWM1200 recipient strain in BTZ medium and incubate the culture at 30°C. It may be necessary to start the culture for MWM1200 a day earlier since it grows slower as compared to *E. coli* strains.
3. For conjugation experiment, the ratio of these three strains is 1:2:6 based on volume (see Note 7).
4. Harvest the overnight cultures by centrifugation and wash them with BTZ medium before combining them together.
5. Spot the pellet containing all three strains onto a nonselective BTZ agar plate supplemented with 0.5% yeast extract.
6. Incubate the plate in a jar or box filled with 25% methane for 3 days at 30°C.
7. Spread the cultures onto BTZ agar plates supplemented with Kan (50 μg/mL) for selection.
8. Place the plates in a jar or box with methane gas mixture and incubate them at 30°C until single colonies with orange color appear.
9. Streak these colonies onto new BTZ plates.
10. Repeat this process to purify the *Methylomonas* exconjugants away from the *E. coli* cells.
11. Culture the selected exconjugants at 30°C in liquid BTZ medium without Kan in serum bottles. Carry out passage of the grown cultures three times.

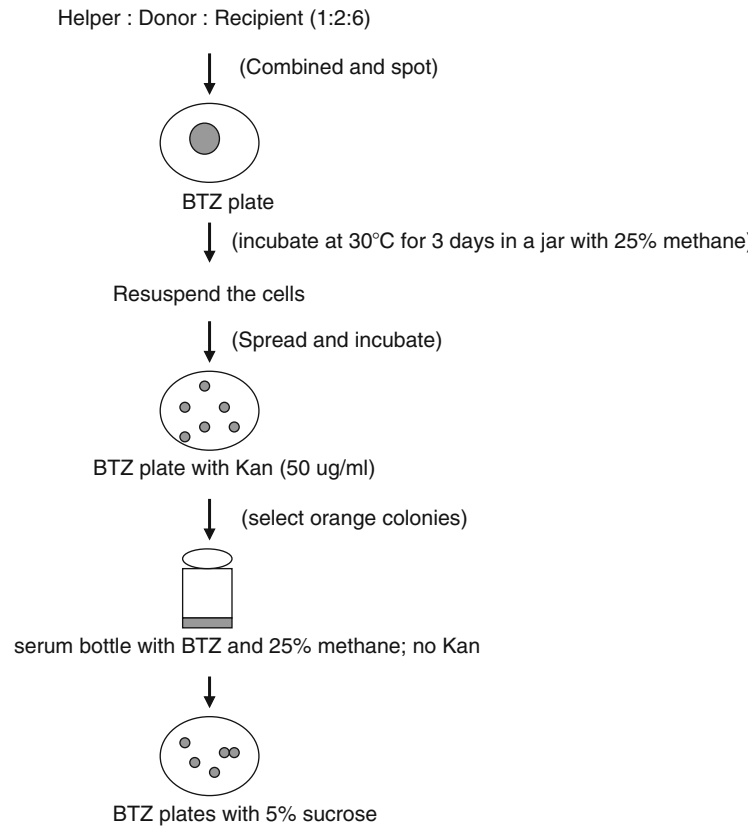

Fig. 2. Outline of procedures for integration of *crt* genes in the chromosome of *Methylomonas* sp. strain 16a (see Subheading 3.2).

12. Plate these cultures on BTZ plates supplemented with sucrose (5% final concentration) (see Note 8).
13. Test the growth of the individual colonies with orange color on BTZ agar plates supplemented with Kan (50 μg/mL).
14. Select strains that are sensitive to Kan.
15. Confirm the integration of the cluster via double crossover with two outside primer sets (see Note 9).
16. Name the selected strain as Orihps333. Extract the canthaxanthin from Orihps333 and analyze the canthaxanthin content by HPLC (8).

3.3. Integration of the crtZ Gene for Astaxanthin Production

The basic strategy and procedure are similar to the steps outlined in Subheadings 3.1 and 3.2.

1. The DNA region containing *crtN*, *ald*, and c*rtNb* genes (see Note 10) is chosen for the integration of *crtZ* gene for astaxanthin biosynthesis.

2. Amplify the 5′ DNA region (upstream) for homologous recombination with primer set G and H, and the 3′ DNA region (downstream) with primers I and J.
3. To amplify the entire region, carry out overlapping PCR with both fragments as the template using the forward primer of the upstream region (primer G) and the reverse primer of the downstream region (primer J).
4. Digest PCR product with XbaI and BglII.
5. Clone the fragment into the integration vector pSUSMK, resulting in the construction of pSUSMKald.
6. Insert the *hps* promoter to the plasmid, which results in the construct pSUSMKaldhps.
7. Amplify the *crtZ* from pDCQ334 plasmid with primers K and L.
8. Clone the *crtZ* gene into pSUSMKaldhps to obtain the plasmid pSUSMKaldhps-crtZ.
9. Transfer the plasmid pSUSMKaldhps-crtZ into the canthaxanthin-producing strain Orihps333 through conjugation as described in Subheading 3.2. The integrated strain obtained is Ax6-5.
10. Extract and analyze the carotenoid content of Ax6-5 by HPLC (8). The strain obtained should produce astaxanthin (see Note 11).

3.4. Integration of an Additional Astaxanthin Gene Cluster

To avoid the unstability of integrated strains, a different astaxanthin biosynthetic gene cluster *crtWZEidiYIB* from pDCQ392 (Table 1) is used for integration into strain Ax6-5. The DNA region *ccp* encoding the cytochrome *C* peroxidase (see Note 12) is chosen as the integration site.

1. Amplify the upstream region including the *ccp* promoter with primers M and N.
2. Amplify the downstream region with primers O and P.
3. Clone the upstream region as an NheI and SpeI fragment into the XbaI site of the integration vector pSUSMK.
4. Clone the downstream region as an EcoRI and BglII fragment, generating the construct pSUSMKccp.
5. Digest the gene cluster *crtWZEidiYIB* as an EcoRI fragment from pDCQ392 and clone it in the EcoRI site of pSUSMKccp.
6. Transfer the resulting plasmid into strain Ax6-5 via conjugation as described in Subheading 3.2. After completing the double crossover selection, name the resulting strain as Ax392 (see Note 13).
7. Analyze the astaxanthin content of Ax392 by HPLC (8).

Although the experiment starts with integrating the canthaxanthin first, direct integration of the astaxanthin clusters in multiple locations is desirable if that is the product of interest. In this experiment, two sets of astaxanthin biosynthetic genes from different organisms are chosen to reduce the likelihood of instability due to homologous recombination.

4. Notes

1. The colorless MWM1200 strain derived from the wild-type *Methylomonas* is used for integration experiments.
2. The integration plasmid pSUKSM has a Kan-resistant marker for selection in MWM1200, an *oriT* region for conjugal transfer, and an *sacB* region for counter selection for double crossovers. The *oriT* region is required for transfer of the plasmid from *E. coli* to recipient bacteria. However, this plasmid does not have regions for replication from broad host range plasmids and thus cannot replicate in *Methylmonas*.
3. For growing the strain in liquid medium, serum bottles with 150- or 500-mL volume are used. The bottles are sealed with PTFE backed butyl rubber stoppers. The gas/liquid ratio in the bottle is at least 8:1 in all experiments to allow the presence of sufficient oxygen and methane gas. Deliver methane with a syringe into the serum bottle so that the gas phase in the serum bottle contains 25% methane by volume.
4. For incubation of agar plates, place plates in a gas jar or gas box. Flush the jar or the gas box with methane/air mixture for 40 min before sealed. The gas mixture consisted of 25% methane and 75% air by volume.
5. The putative origin of replication is next to the *dnaA* gene which encodes DnaA, a replication initiation factor. A region close to the origin of replication is chosen for integration with the assumption that the integrated gene will be expressed at higher level due to the increase in copy number during replication. However, this assumption has not been proven. For homologous recombinations, the two homologous regions flanking the DNA to be integrated have to be around 0.8 kb or longer to obtain good efficiency of recombination.
6. The *hps* gene encodes the 3-hexulose-6-phosphate synthase, which catalyzes the first step in the RuMP pathway for formaldehyde assimilation. The *hps* promoter is used to express the canthaxanthin gene cluster. This is one of the genes that is highly expressed based on DNA microarray experiment (9).

7. We normally use 5 mL of helper strain, 10 mL of the donor strain (with the integration vector), and 30 mL of Kan-sensitive recipient *Methylomonas* host.
8. Strains with single crossover still have the vector backbone which contains the *sacB* gene and thus are sensitive to sucrose. The purpose of passage is to enrich those that have undergone double crossover. Those strains growing on sucrose will be either the parent strain or the strains with integration via double crossover. The sucrose has to be freshly prepared.
9. Four primers are needed for confirmation. Design two outside primers based on genomic regions outside the integration region and two internal primers in opposite direction based on the *crt* region.
10. This DNA region is involved in the native pigment production and has been shown to be nonessential for cell growth. Mutations in this region render *Methylomonas* sp. strain 16a colorless and thus useful for the introduction of carotenoid gene cluster for visual selection based on color formation.
11. We observed that the strain with a single copy of the *crtZ* gene produced both astaxanthin and the mono-ketolated intermediate adonixanthin. The astaxanthin consisted of 64% of the total carotenoid.
12. The *ccp* region has been shown to support a high level of expression of *crt* genes (13).
13. In strain Ax392, the expression of *crtWZEidiYIB* gene cluster is under the control of the native promoter for the *ccp* gene. This final strain should produce a higher amount of astaxanthin. We observed that the strain with a second complete astaxanthin cluster produces up to 95% of the total carotenoid in the culture as astaxanthin. Among the different forms of astaxanthin isomers, the all *E*-isomer is 89%. In addition, the strain proved to be very stable under fermentation conditions. To achieve a high level of astaxanthin production, it is important to maintain a high oxygen level. Lower oxygen level often results in accumulation of intermediates and thus lower selectivity for astaxanthin production for engineered strains (14).

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