

Use of Transposon Promoter-Probe Vectors in the Metabolic Engineering of the Obligate Methanotroph *Methylomonas* sp. Strain 16a for Enhanced C₄₀ Carotenoid Synthesis^{∇†}

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The recent expansion of genetic and genomic tools for metabolic engineering has accelerated the development of microorganisms for the industrial production of desired compounds. We have used transposable elements to identify chromosomal locations in the obligate methanotroph *Methylomonas* sp. strain 16a that support high-level expression of genes involved in the synthesis of the C₄₀ carotenoids canthaxanthin and astaxanthin, with three promoterless carotenoid transposons, five chromosomal locations—the *fliCS*, *hsdM*, *ccp-3*, *cysH*, and *nirS* regions—were identified. Total carotenoid synthesis increased 10- to 20-fold when the carotenoid gene clusters were inserted at these chromosomal locations compared to when the same carotenoid gene clusters were integrated at neutral locations under the control of the promoter for the gene conferring resistance to chloramphenicol. A chromosomal integration system based on sucrose lethality was used to make targeted gene deletions or site-specific integration of the carotenoid gene cluster into the *Methylomonas* genome without leaving genetic scars in the chromosome from the antibiotic resistance genes that are present on the integration vector. The genetic approaches described in this work demonstrate how metabolic engineering of microorganisms, including the less-studied environmental isolates, can be greatly enhanced by identifying integration sites within the chromosome of the host that permit optimal expression of the target genes.

The development of new genetic and genomic tools has enhanced the ability to metabolically engineer a diverse population of microorganisms for the production of a variety of compounds. This capability has been extended to include the obligate methanotroph *Methylomonas* sp. strain 16a (ATCC PTA-2402). *Methylomonas* sp. strain 16a is often distinguished by its pink color, which has been found to be due to the production of C₃₀ carotenoids, including 4,4'-diapolycopene-4-oic acid, 4,4'-diapolycopene-4,4'-dioic acid, and the ester forms of these molecules (22). Commercial applications for the C₃₀ class of carotenoids have not been discovered. *Methylomonas* sp. strain 16a, however, is an attractive host for the production of the highly diverse and commercially valuable C₄₀ class of carotenoids. Like many other microorganisms found in nature, *Methylomonas* sp. strain 16a contains the isoprenoid pathway genes, which are also necessary for the synthesis of the C₄₀ carotenoids (5, 14, 16, 17, 20). C₄₀ carotenoids, including lycopene, β-carotene, canthaxanthin, and astaxanthin, are used as food supplements, as well as colorants, in the food and feed markets of the nutraceutical, poultry, and aquaculture industries (5, 16).

Metabolic engineering of *Methylomonas* for the production

of C₄₀ carotenoids requires two essential genetic manipulations. The first required step is to block the ability of *Methylomonas* to produce C₃₀ carotenoids via gene deletions. Therefore, the next metabolic engineering step is to introduce the C₄₀ carotenoid biosynthesis genes into pigmentless strains of *Methylomonas* (14, 15, 16, 17, 19). These genes include *crtE*, *crtY*, *crtI*, *crtB*, *crtW*, and *crtZ* (Fig. 1). Expression of the *crtE* gene product (geranylgeranyl pyrophosphate synthase) is the first committed step in the synthesis of C₄₀ carotenoids. Two farnesyl pyrophosphate 20-carbon molecules are condensed by a geranylgeranyl pyrophosphate synthase (CrtE) to form the 40-carbon molecule geranylgeranyl pyrophosphate. In turn, a phytoene synthase (CrtB) converts geranylgeranyl pyrophosphate into phytoene, a phytoene dehydrogenase (CrtI) converts phytoene into lycopene, and a lycopene cyclase (CrtY) converts lycopene into β-carotene. The β-carotene modification gene *crtW* is required for the synthesis of canthaxanthin, and the further expression of *crtZ* is needed for the production of astaxanthin. Since both CrtW (β-carotene hydroxylase) and CrtZ (β-carotene ketolase) can use β-ionone rings as a substrate, many different C₄₀ intermediates can be formed, depending on which enzyme acts first and which end of the molecule is modified first (Fig. 1) (15).

A desirable trait of a fermentation production microorganism includes the stable integration of heterologous genes into the host's chromosome. This alleviates the use of antibiotics that are required to exert selective pressure for plasmid maintenance during fermentation. In addition, the regulatory approvals for many commercial fermentation products are more

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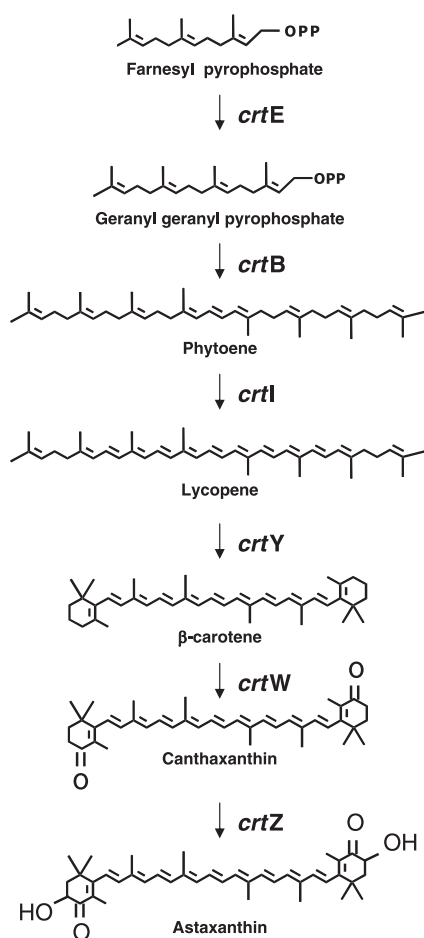


FIG. 1. Biosynthesis of C_{40} carotenoids. Production of C_{40} carotenoids (phytoene, lycopene, β -carotene, canthaxanthin, and astaxanthin) requires the addition of several carotenoid genes (*crtE*, *crtB*, *crtI*, *crtY*, *crtW*, and *crtZ*). OPP, octaprenyl pyrophosphate.

favorable if antibiotic resistance genes are not present in the production strains.

Achieving optimal transcription and translation of foreign genes integrated into the host's chromosome is important in meeting desired product yields. Since gene expression can be influenced by promoter strength, promoter probe vectors have been used in many studies to identify promoter sequences for the heterologous expression of genes of interest (2, 3, 4, 7, 18). One method of using promoter selection/probe vectors is to insert DNA fragments into a polylinker region that precedes a promoterless reporter gene. DNA fragments that turn on the expression of the reporter gene contain promoter sequences (2, 3, 8). Another method involves the use of promoter probe transposons to insert promoterless reporter genes randomly into the chromosome, which can often generate transcriptional fusions, as well as insertional mutations (4, 9, 21, 23). Additionally, promoter probe vectors have been used to identify resident promoters that allow the expression of single-copy of recombinant genes in the chromosome (8). For example, *Lactobacillus plantarum* has been engineered for the expression of α -amylase and levanase, in which expression and secretion signals were isolated by a probe vector approach. In addition,

a two-step integration method was used to integrate the signals into the *L. plantarum* chromosome without the introduction of resistance markers or vector sequences (6).

The genetic technique described in this paper uses a promoterless transposable element based on the Tn5 transposon. Promoterless carotenoid gene clusters, whose expression is required for the synthesis of C_{40} carotenoids, were inserted between the Tn5 transposon ends. The uniqueness of this promoterless-transposon approach is the ability to identify resident promoter regions that support the optimal expression of a carotenoid gene cluster that is present as a single copy without knowing a priori where in the *Methylomonas* chromosome the transposon will be inserted. We will demonstrate in this report that the chromosomal location in which the C_{40} carotenoid gene clusters are integrated greatly influences the level of carotenoid production. We will also describe how this promoterless-transposon method has permitted the identification of several chromosomal regions that support ~10- to 20-fold greater total carotenoid production. Some of the chromosomal locations identified also supported an approximately twofold greater level of total carotenoid production than when the carotenoid gene cluster was expressed on a high-copy-number plasmid. Furthermore, in this report we describe a chromosomal integration method that allows the markerless integration of C_{40} carotenoid genes into chromosomal regions that enhance astaxanthin synthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. The *Methylomonas* strains were grown in BTZ medium (see supplemental material) in serum-stoppered bottles (Wheaton Scientific) containing 25% methane. The *Escherichia coli* strains used for cloning were grown in LB medium containing the appropriate antibiotics.

Modification of transposon delivery vector pUTmini-Tn5gfp. The starting material used in the construction of a transposon delivery vector included the base vector pUTmini-Tn5gfp, which was modified via removal of the gene conferring resistance to tetracycline and the gene encoding the green fluorescent protein (*gfp*) (12). Concurrent with the removal of these features, a polylinker encoding multiple restriction sites was introduced to facilitate subsequent cloning of antibiotic resistance markers and carotenoid genes (see supplemental material). The use of the XmaI restriction sites on the polylinker and on the base vector was a part of the cloning strategy for the bidirectional cohesive end ligation of the polylinker containing multiple cloning sites. Invitrogen (<http://www.invitrogen.com/>) synthesized the 58-nucleotide oligomer polylinker and its reverse complement. The forward sequence of the polylinker was 5'-AATTCGCGGAC TAGTACGCGTGCGGCCCGCCCATGGCATATGTTCCGAACCCGGGTAC C3'. The reverse complement sequence of the polylinker was 5'-GGTACCCGG GTTCCGAACATATGCCATGGGCGGCCGCACGCGTACTAGTCCCGGGA ATT3'. The two polylinker DNA molecules were annealed by mixing equimolar quantities (20 μ l of 100-pmol/ μ l stock solutions) in a total volume of 100 μ l of water and by heating the mixture to 100°C for 5 min, followed by gradual cooling to 40°C over a 20-min period. Afterward, the annealed mixture was transferred to an ice bath, leading to the formation of double-stranded DNA. The double-stranded polylinker was digested with XmaI and cloned into XmaI-digested base vector pUTmini-Tn5gfp. In order to accommodate the vector's R6K origin of replication, electroporation-competent *E. coli* SY327 (λ pir) was used as the host strain in the transformations. Subsequently, 50 μ l of the transformation mixture was plated on LB containing ampicillin at 100 μ g/ml and incubated overnight at 37°C. Plasmid DNA was isolated from transformed *E. coli* cells, and the plasmid DNA was analyzed via diagnostic restriction digestions with SpeI/NheI to reveal correct clones with expected fragment sizes of 1.1 kb and 4.2 kb. The orientation of the insert DNA was determined via DNA sequencing with two DNA sequencing primers, pUTmTn5/Seq.F (5'-GCACGATGAAGAGCAGAAGTTATC3') and pUTmTn5/Seq.R (5'-AACACTTAACGGCTGACATGG3'). This versatile transposon delivery vector was named pUTmTn5- (see supplemental material).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
MWM1200	<i>Methylomonas</i> 16a Δ promoter <i>crt</i> cluster Δ <i>crtNc</i>	This work
MWM1500	MWM1200 Δ <i>glgA</i>	This work
MCS003	MWM1200/pDCQ307	This work
Cat333	MWM1200 <i>Phps-crt333::ori crtWZ::ald</i>	This work
MWM1500	MWM1200 Δ <i>glgA</i>	This work
MCIS1100	MWM1200 <i>crt333::tig ΔglgA</i>	This work
MCIS1701	MWM1200 <i>Tn334::ccp-3</i>	This work
MCIS1702	MWM1200 <i>Tn334::mst1341</i>	This work
MCIS1703	MWM1200 <i>Tn334::mst2848</i>	This work
MCIS1801	MWM1500 <i>Tn334::fliS</i>	This work
MCIS1804	MWM1500 <i>Tn334::ccp-3</i>	This work
MCIS1807	MWM1500 <i>Tn334::mst1132</i>	This work
MCIS2201	MCIS1100 <i>Tn341::fliS</i>	This work
MCIS2203	MCIS1100 <i>Tn341::fliC</i>	This work
MCIS2601	MWM1200 <i>Tn377::mst2848</i>	This work
MCIS2602	MWM1200 <i>Tn377::mst1132</i>	This work
MCIS3001	MWM1200 Δ C ₃₀ cluster <i>Tn334::ccp-3</i>	This work
MCIS4101	MCIS3001 <i>Tn377::nirS</i>	This work
SY327 (λ <i>pir</i>)	Δ (<i>lac-pro</i>) <i>argE</i> (Ap) <i>recA56 nalA</i> R ^r (λ <i>pir</i>)	13
Plasmids		
pBHR1	pBBR1 derivative; Kn ^r Cm ^r <i>mob</i> ⁺ <i>oriT</i> ⁺	1
pDCQ307	pBHR1; <i>crtWZEYIB</i> Kn ^r	This work
pDCQ334	pBHR1; <i>crtWZEiYIB</i> Kn ^r	This work
pDCQ341	pBHR1; <i>crtWEYIB</i> Kn ^r	This work
pDCQ343	pBHR1; <i>crtWZEYIB</i> Kn ^r	This work
pDCQ377	pBHR1; <i>crtWZEiYIB</i> Kn ^r	This work
pDCQ392	pBHR1; <i>crtWZEiYIB</i> Kn ^r	This work
pUTmini-Tn5 <i>gfp</i>	Mini-Tn5 <i>gfp</i> reporter <i>mob</i> ⁺ Ap ^r Tc ^r	11
pUTmTn5-	Mini-Tn5 <i>mob</i> ⁺ Ap ^r MCS	This work
pUTmTn5-334Kn	pUTmTn5-; <i>crtWZEiYIB</i> Kn ^r	This work
pUTmTn5-341Kn	pUTmTn5-; <i>crtWZEYIB</i> Kn ^r	This work
pUTmTn5-377Kn	pUTmTn5-; <i>crtWZEiYIB</i> Kn ^r	This work
pGP704	Ap ^r <i>ori</i> R6K <i>mob</i> RP4 MCS M13tg131	13
pGP704- <i>sacB</i>	pGP704; <i>npr_{ss}-lvs</i> (<i>sacB</i>)	This work
pBE83	Ap ^r Cm ^r <i>npr_{ss}-lvs</i> (<i>sacB</i>)	18

Plasmid pUTmTn5- was the vector used in the construction of the Tn334, Tn341, and Tn377 promoterless carotenoid transposons.

Construction of transposon delivery vector pUTmTn5-334Kn. By standard cloning methods, the promoterless *crt334* carotenoid biosynthetic pathway gene cluster was subcloned from pDCQ334 into pUTmTn5- by digestion of both vectors with BstBI and SpeI. The 7.4-kb DNA fragment contained the gene cluster (*crtW*, *crtZ*, *crtE*, *idi*, *crtY*, *crtI*, and *crtB*) from pDCQ334 and the 5.2-kb DNA fragment encompassing the linearized pUTmTn5- vector (see supplemental material). The vector was named pUTmTn5-334.

Subsequently, the kanamycin antibiotic resistance gene from the EZ::TN<Kan-2> (Epicenter) transposon was ligated between the transposon ends. PCR amplification of the EZ::TN<Kan-2> kanamycin resistance gene was carried out with PCR primers KnAvrIIKpnIBstBIR2 (5'ATGCTTCGAACGGGTACCTAGGATGCGTGATCTGATCC3') and KnBstBIF (5'TGGCTTCGAACGATGAATTGTGTCTC3').

The BstBI- and AvrII-digested DNA fragment containing the kanamycin resistance gene was ligated into TOPO vector pCR2.1 (Invitrogen), resulting in pCR2.1-Kn. Subsequently, the kanamycin DNA fragment was excised via digestion with BstBI/AvrII and ligated with the BstBI/AvrII-digested pUTmTn5-334 vector DNA. The ligation mixture was used to transform *E. coli* SY327 (λ *pir*) cells. Afterwards, the transformation mixture was plated onto LB agar plates supplemented with kanamycin at 25 μ g/ml and plasmid DNA was isolated from several of the resulting colonies. The pUTmTn5-334Kn candidates were confirmed by digestion with XhoI and NotI.

Construction of transposon delivery vector pUTmTn5-343Kn. Customary molecular cloning techniques were used to construct pUTmTn5-343Kn. Four of the promoterless *crt343* carotenoid genes (*crtE*, *crtY*, *crtI*, and *crtB*) were taken from pDCQ343 by digestion with BstBI and SpeI and then ligated with BstBI/SpeI-digested pUTmTn5 vector DNA, generating pUTmTn5-343EYIB.

The *crtW* and *crtZ* genes were amplified from pDCQ343 with PCR primers p343*crtZ*SpeI (5'TACCCACTAGTAAGGAGGAATAAACCATGACCG3') and p343*crtW*SpeI (5'GGTTGGTACTAGTTCAGGC3'). The PCR product was digested with SpeI and ligated into the SpeI site of pUTmTn5-343EYIB. This plasmid was referred to as pUTmTn5-343, which was further modified by the addition of the chloramphenicol resistance (Cm^r) gene. The Cm^r gene was amplified from pBHR1 with PCR primers CmAvrIIKpnIBstBIR (5'ATGCTTCGAACGGGTACCTAGGCGTTTAAGGGCACCAATAAC3') and CmBstBIF (TGGCTTCGAATACCTGTGACGGAAGATC3'), and the AvrII-and-BstBI DNA fragment was ligated into the AvrII/BstBI-digested pUTmTn5-343 vector, giving rise to pUTmTn5-343Cm. Subsequently, the Cm^r gene was removed from the vector DNA by digestion with AvrII/BstBI and the Kn^r gene was ligated into the same sites of the gutted vector. Thus, the Kn^r version of this vector was made and named pUTmTn5-343Kn.

Construction of transposon delivery vector pUTmTn5-341Kn. Transposon delivery vector pUTmTn5-341Kn was constructed by removing the *crtZ* gene from pUTmTn5-343Kn. This was accomplished by digesting pDCQ341 and pUTmTn5-343Kn with BsrGI and AatI. The pUTmTn5-343Kn DNA fragment contained a partial *crtW* gene, a partial *crtI* gene, an intact *crtB* gene, and an intact Kn^r gene. The pDCQ341 DNA fragment contained a partial *crtW* gene, a partial *crtI* gene, an intact *crtE* gene, and *crtY*. The two DNA fragments were ligated together, forming pUTmTn5-341Kn.

Transposition of Tn334Kn into the *Methylomonas* genome. The promoterless carotenoid transposon delivery vector was transferred into *Methylomonas* via triparental conjugation. Specifically, the following strains were used as the recipient, donor, and helper, respectively: *Methylomonas* strain MWM1500, *E. coli* SY327 (λ *pir*) containing the promoterless carotenoid transposon delivery vector (pUTmTn5-334Kn), and *E. coli* containing helper plasmid pRK2013 (ATCC 37159). The resulting colonies were screened to identify those having the darkest

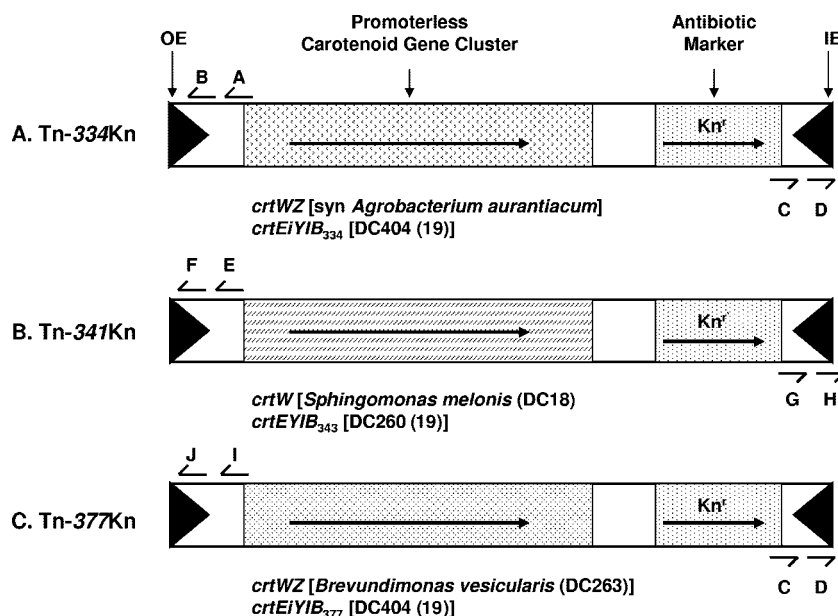


FIG. 2. Promoterless carotenoid transposons used in this study. Three carotenoid transposons were constructed with the carotenoid genes from various carotenoid clusters and are represented by the larger shaded boxes. The smaller shaded boxes represent the kanamycin resistance gene. The OE of transposable element Tn5 is represented by the rightward-pointing triangle. The IE of transposable element Tn5 is represented by the leftward-pointing triangle. The arrows labeled B, C, F, G, and J show the locations of the single-primer PCR DNA primers. The arrows labeled A, D, E, H, and I show the locations of the DNA sequencing primers.

color. This method was also used for the other promoterless carotenoid transposon delivery vectors.

Identification of the carotenoid transposon insertion sites in the *Methylomonas* genome. Two different approaches were used to determine the locations of the transposons (Tn334Kn, Tn341Kn, and Tn377Kn) within the *Methylomonas* genome. A single-primer PCR method was performed with PCR and sequencing primers specific for the ends of the transposon (8). Another methodology used to identify transposon insertion sites was direct sequencing of the *Methylomonas* genomic DNA with DNA primers specific for the ends of the transposon. For a list of the DNA primers used in both methods, see the supplemental material. Primers A and C, primers E and G, and primers I and C were used to PCR amplify chromosomal regions flanking the Tn334, Tn341Kn, and Tn377 transposon insertion sites, respectively. Following PCR amplification of the transposon insertion region, PCR products were processed to remove the PCR primers with the Qiaquick Nucleotide Removal Kit (<http://www1.qiagen.com>). The corresponding sequencing primers were used to sequence the PCR fragment, and the sequence information was used to determine the transposon-chromosome junction site. Sequencing primer B was used to sequence the “A” PCR product generated from the insertion of the Tn334Kn transposon. Sequencing primer J was used to sequence the “I” PCR products generated from the insertion of the Tn377Kn transposon. Sequencing primer F was used to sequence the “E” PCR product, and sequencing primer H was used to sequence the “G” PCR product for the Tn341Kn transposon. Sequencing primer D was used to sequence to the other end of the A/C and I/C PCR products generated from the insertion of the Tn334Kn and Tn377Kn transposons, respectively (Fig. 2).

Construction of the transposon delivery vector to generate pUTmTn5-377Kn. The transposon delivery vector pUTmTn5-377Kn was constructed by removing, via digestion with XmaI and NcoI, the promoterless carotenoid cluster from the pUTmTn5-334Kn transposon delivery vector and replacing the DNA fragment with the carotenoid gene cluster from pDCQ377 via digested with BspEI and NcoI. The ligation of the two DNA fragments generated the pUTmTn5-377 transposon delivery vector.

Direct sequencing of *Methylomonas* genomic DNA. The direct sequencing of genomic DNA was performed by the following method. The sequencing reaction mixtures consisted of 3 μ g of purified genomic DNA, 16 μ l of BigDye v3.1 sequencing reagent (PN no. 4337457; Applied Biosystems), 3 μ l of 10 μ M primer, 1 μ l of Thermofidase (Fidelity Systems), and 12 μ l of molecular biology grade water. By a thermal-cycling procedure, the sequencing reactions were carried out by heating the samples for 3 min at 96°C, followed by 200 cycles of 95°C for 30 s, 55°C for 20 s, and 60°C for 2 min, and then storing the samples at

4°C. The unincorporated dideoxynucleoside triphosphates were removed prior to sequencing. For each sequencing reaction mixture, a total of 40 μ l was transferred to one well of a prespun 96-well cleanup plate. The plate was spun for 5 min at 5,000 $\times$ g. The cleaned up reactions were placed directly onto an Applied Biosystems 3700 DNA sequencer and sequenced by automatic base calling.

Removal of the kanamycin resistance gene after insertion into the *Methylomonas* genome. The kanamycin resistance gene present in the promoterless carotenoid transposons was removed from the *Methylomonas* genome via homologous recombination with the pGP704-*sacB* integration vector. In general, an approximately 1-kb region on each side of the kanamycin resistance gene was PCR amplified and cloned into the pGP704-*sacB* vector. Through homologous recombination and selection on BTZ medium containing 5% sucrose, cells that have lost the vector sequences are detected. The sucrose-resistant colonies are screened for those having lost the kanamycin resistance gene by PCR methodology with DNA primers specific for the region.

In the case of MCIS3000, which had the Tn334 transposon inserted into the *ccp-3* region (Tn334Kn::*ccp-3*), the kanamycin resistance gene was removed from the *Methylomonas* chromosome via homologous recombination with the pGP704-*sacB* vector containing an arm consisting of DNA from *crtB* and an arm consisting of DNA from the *ccp-3* gene. This vector was constructed with the following PCR primers. The primers for the *crtB* arm were 334kndel R1 (5'TG ATTCTAGATTCTAGCGCGGGCGCTGCCAGAG3') and 334kndel F1 (5'G CCTAGATCTGGTTGATAACCTCTACCTGGTTCG3'). The primers for the *ccp-3* arm were Tn5-334Kn1200-1 3'R (5'CCCCGGTATATCGAGGTTATCA TCGTG3') and Tn5-334Kn1200-1 3'F (5'TCTAGAAGGTGCCAGCTTGCG TAACGTAG3'). The *crtB* arm PCR product was ligated into the BglII and XbaI sites of the pGP704-*sacB* vector, generating pGP704-SacBkndel1, which was subsequently digested with XbaI and XmaI and ligated with the *ccp-3* arm, generating pGP704SacB1200-1kndel2. This vector was conjugated into MCIS3000. Cells that had undergone a single-crossover event were selected by plating onto BTZ medium containing ampicillin (50 μ g/ml). Single colonies were then inoculated into BTZ medium without the addition of ampicillin and grown to saturation. Dilutions of the dense cultures were plated onto BTZ medium containing 5% sucrose, which selected for cells that no longer harbored the *sacB* gene. PCR methodology was used to identify cells that had lost their kanamycin resistance gene via double-crossover events. Wild-type cells produced a PCR fragment that was ~3.0 kb in size, and the kanamycin deletion cells gave rise to an ~2.0-kb PCR fragment. The cells were confirmed no longer to possess the kanamycin resistance gene by demonstrating lack of growth on medium containing kanamycin. The resulting strain was named MCIS3001.

Total carotenoid titer evaluation and percent astaxanthin selectivity of the carotenoid transposon insertion strains. The carotenoid titers for the transposon insertion strains were calculated by determining the amount of carotenoid (milligrams of carotenoid[s] per gram of dry cell weight [DCW]). After cultivating the *Methylomonas* astaxanthin- or canthaxanthin-producing strains in 50 ml of BTZ medium in a 500-ml capped serum bottle containing 25% methane, 20 ml of the culture was used for carotenoid extraction and 20 ml of the culture was used to determine the DCW.

For carotenoid extractions, the cells were concentrated in a 50-ml polypropylene tube. Following removal of the supernatant, approximately 0.5 ml of 0.1-mm glass beads was added to the pellet. To this mixture, 1 ml of methanol and 1.5 ml of dichloromethane were added and the mixture was vortexed for approximately 2 min (until the cells were broken). The cellular debris was removed by centrifugation at 8,000 rpm for 10 min. The supernatant was transferred to a new 50-ml polypropylene tube, and the extracted carotenoids were dried under nitrogen until all of the liquid evaporated. The dried pellets were resuspended in 90 μ l of chloroform plus 1,910 μ l of hexane. The solution was filtered with a 0.2- μ m-pore-size Teflon filter (Pall Gelman Acrodisc 13 CR polytetrafluoroethylene syringe filter) to remove any particles. The filtered carotenoid solution was analyzed via high-pressure liquid chromatography against standards of known concentrations.

To determine the DCW of the *Methylomonas* carotenoid-producing strains, the cultures were applied to a 47-mm polycarbonate Whatman Nuclepore Track-Etch membrane (47-mm diameter and 0.2- μ m pore size) to collect the *Methylomonas* cells. A vacuum was applied to remove the liquid. The filter was allowed to dry overnight in a 105°C oven. The DCW was calculated by subtracting the weight of the filter alone from the weight of the filter plus the cells.

To determine the percentage of astaxanthin produced in the carotenoid-producing strains, the carotenoids were extracted by the procedure described above. However, the cultures were cultivated by growing 10 ml of the carotenoid-producing strains in BTZ medium in a 500-ml capped serum bottle containing 25% methane.

Nucleotide sequence accession numbers. The nucleotide sequence accession number for the β -carotene ketolase and the β -carotene hydroxylase genes from *Paracoccus* sp. strain N81106 is D58420. The nucleotide sequence accession number for the *crtW* and *crtZ* genes from *Brevundimonas vesicularis* DC263 is DQ309446. The nucleotide sequence accession number for the *nirS* gene is AX268051.

RESULTS

Expression of a canthaxanthin biosynthetic gene cluster on a multicopy plasmid and in the *Methylomonas* chromosome. *Methylomonas* sp. strain 16a (ATCC PTA-2402) naturally produces C_{30} carotenoids (4,4'-diapolycopene-4-oic acid, 4,4'-diapolycopene-4,4'-dioic acid, and other derivatives), which accounts for its pink pigment (22). Carotenoid expression plasmid pDCQ333, which contains the genes necessary for canthaxanthin expression, was transferred via conjugation to MWM1200, a pigmentless strain constructed by disrupting the promoter that drives the expression of the native C_{30} carotenoid cluster. *Methylomonas* cells containing pDCQ333 were orange, indicating expression of the C_{40} carotenoid genes. The carotenoid titer was ~ 1.0 mg/g of DCW (see supplemental material). This result demonstrates that C_{40} carotenoid genes can be exogenously expressed in *Methylomonas* sp. strain 16a. Our goal was to construct carotenoid-producing *Methylomonas* strains that are stable and do not contain any antibiotic resistance genes. This was accomplished by integrating the carotenoid genes into the *Methylomonas* genome.

The canthaxanthin biosynthetic gene cluster from pDCQ333 was integrated into the genome of MWM1200, resulting in the construction of Cat333. The level of carotenoid production in this strain was about 0.1 mg/g of DCW (see supplemental material). This was $\sim 90\%$ lower than the levels detected when the carotenoid genes were expressed from the same promoter (*Pcat*) present on plasmid pDCQ333. This indicated that it was

necessary to identify chromosomal locations that support enhanced expression of the carotenoid genes.

Construction of promoterless carotenoid transposable elements to identify highly expressed chromosomal regions in the *Methylomonas* genome. Different carotenogenic gene clusters (*crt334*, *crt341*, and *crt377*) involved in the biosynthesis of C_{40} carotenoids were used to make promoterless carotenoid transposons. Although the desired C_{40} carotenoids canthaxanthin and astaxanthin were synthesized, other C_{40} intermediates were also detected.

Three different promoterless carotenoid transposons were constructed, each containing carotenoid genes from different bacterial sources (19). These preassembled gene clusters were taken from carotenoid expression plasmids pDCQ334, pDCQ341, and pDCQ377. The promoterless carotenoid gene clusters were inserted between the inside ends (IE) and outside ends (OE) of the mini-Tn5 transposon (see supplemental material). In addition to the promoterless carotenoid gene cluster, each transposable element also contains a kanamycin resistance gene between the IE and the OE. Addition of the carotenoid gene clusters and the kanamycin antibiotic resistance gene to pUTmTn5– resulted in the construction of transposon delivery vectors containing the Tn334Kn, Tn341Kn, and Tn337Kn transposons (see the supplemental material).

Use of in vivo transposition to screen for chromosomal regions that support high-level expression of C_{40} carotenoids. In vivo transposition with the promoterless carotenoid transposable elements was used to survey the *Methylomonas* genome for chromosomal regions that support elevated carotenoid gene expression. The *Methylomonas* host for the transposition reaction was strain MWM1200 or MWM1500. The sources of the carotenoid transposons were pUTmTn5-334Kn, pUTmTn5-341Kn, and pUTmTn5-377Kn. The transposable elements were mobilized into *Methylomonas* via conjugation. The transposase is located outside the transposon ends; thus, the carotenoid transposons move only once and insertion into the *Methylomonas* genome is stable.

Thousands of *Methylomonas* colonies containing Tn334Kn insertions were detected in a single conjugation. Approximately 9% of the colonies had pigments in various shades of orange ranging from pale orange to dark reddish orange. The colonies having the darkest hues were selected for further analysis.

Identification of the carotenoid transposons' insertion sites in the *Methylomonas* genome. The locations of the carotenoid transposons (Tn334, Tn341, and Tn377) in the *Methylomonas* genome were determined by PCR methodology and DNA sequencing as previously described (8). The sequencing results were used to search for homologous sequences within the *Methylomonas* genome (Jean Francois Tomb, personal communication). Analysis of DNA sequence information obtained at the junctions of the transposon ends and the *Methylomonas* chromosome revealed that the transposition events were clustered in four different chromosomal regions. None of the carotenoid transposons, however, were inserted into identical chromosomal sites. As expected, all of the transposon sites were downstream of putative promoters. Three of the insertion sites were located in the *fliC* and *fliS* genes (Fig. 3). The sizes of the *fliC* and *fliS* genes are 834 and 396 nucleotides, respectively. The *fli* operon in *Methylomonas* has homology to genes known to encode flagellin (FliC) and is involved in flagellin

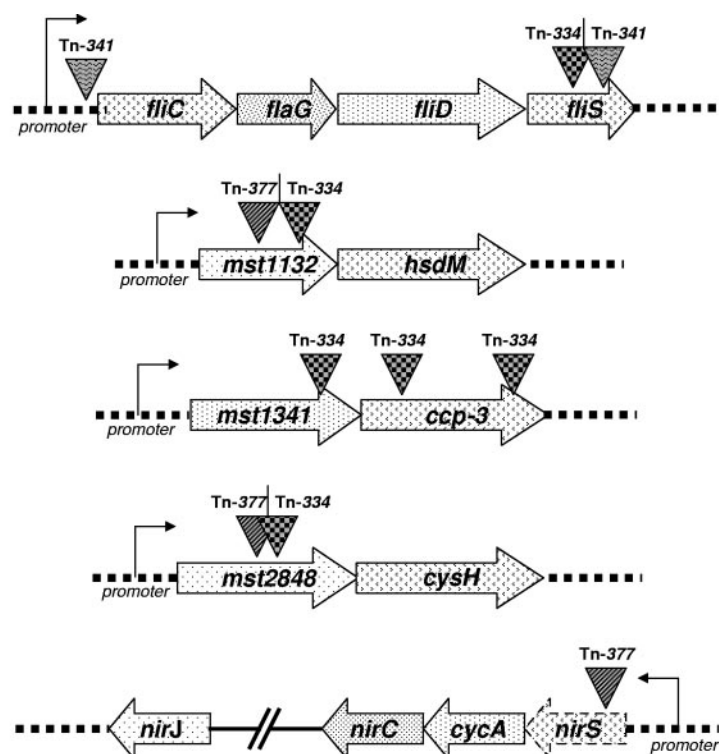


FIG. 3. The insertion sites of carotenoid genes within the *Methylobacterium* genome influence high-level carotenoid production.

export (10). The Tn341 transposon was inserted immediately upstream (four nucleotides) of the *fliC* gene, as well as in the 3' end of the *fliS* gene. DNA sequence information revealed that the Tn334 transposon was also inserted within the *fliS* gene (Fig. 3).

Another region of the *Methylobacterium* chromosome that received multiple insertions that supported enhanced carotenoid gene expression was the *hsdM* region. The *hsdM* gene encodes an enzyme involved in modification of type II restriction systems. The hypothetical gene *mst1132*, located immediately upstream of *hsdM*, was disrupted by two different carotenoid transposons (Tn377 and Tn334) (Fig. 3).

Transposon insertions within the cytochrome *c* peroxidase region were also detected multiple times during the screening to identify chromosomal locations that enabled enhanced expression of the carotenoid clusters. In a Tn334 transposition reaction, the transposable element was inserted within the cytochrome *c* peroxidase gene (*ccp-3*) twice at different locations. Tn334 insertions were found near both the 5' and 3' ends of the *ccp-3* gene. In addition, the Tn334 transposon was found to be inserted into the gene upstream of *ccp-3* (*mst1341*), near its 3' end (Fig. 3).

Improved carotenoid expression was also observed when carotenoid transposons Tn377 and Tn334 were inserted into the hypothetical gene (*mst2848*) located immediately upstream of the *cysH* gene, which encodes phosphoadenosine phosphosulfate reductase (Fig. 3). The size of the hypothetical *mst2848* gene is 1,083 nucleotides, and the transposon insertions were located about 12 nucleotides apart, near the middle of the gene.

The *nirS* region was the fifth chromosomal location identi-

fied that supported elevated carotenoid gene expression. The *nirS* operon is composed of 13 genes, many of which are involved in the function of nitrite reductase. The *nirS* gene is 1,581 nucleotides long, and the Tn377 transposon disrupted the *nirS* gene near its 5' end (Fig. 3). The *nirS* region was identified during a genetic screen in which the *Methylobacterium* host strain already contained the *crt334* gene cluster integrated at the *ccp-3* region (Table 2).

The level of C₄₀ carotenoid expression in *Methylobacterium* is significantly impacted by chromosomal location. A group of carotenoid-producing strains whose colony colors were the darkest on the basis of visual inspection were selected for

TABLE 2. Analysis of carotenoid production by *Methylobacterium* strains

Strain	Transposon(s)	Open reading frame	Insertion site/size of gene ^a	Carotenoid titer ^b
Cat333 ^c	<i>crt333</i> gene cluster		<i>ald</i> region	~0.1
MCIS2203	Tn341	<i>fliC</i>	~4/834	~1.0
MCIS1801	Tn334	<i>fliS</i>	140/396	~2.0
MCIS2201	Tn341	<i>fliS</i>	274/396	~1.1
MCIS2602	Tn377	<i>mst1132</i>	579/2,151	~0.5
MCIS1807	Tn334	<i>mst1132</i>	1302/2,151	~1.3
MCIS1702	Tn334	<i>mst1341</i>	120/816	~1.9
MCIS1804	Tn334	<i>ccp-3</i>	590/2,355	~1.1
MCIS1701	Tn334	<i>ccp-3</i>	1898/2,355	~1.7
MCIS2601	Tn377	<i>mst2828</i>	446/1,521	~0.6
MCIS1703	Tn334	<i>mst2828</i>	488/1,521	~1.7
MCIS4101	Tn334, Tn377	<i>nirS</i>	377/1,581	~0.8

^a The sizes of genes were measured in nucleotides.

^b Total carotenoid titers were measured in milligrams per gram of DCW.

^c *Methylobacterium* sp. strain Cat333 was constructed by integrating the *crt333* gene cluster for canthaxanthin production into the *ald* region with the C₃₀ carotenoid gene cluster.

further evaluation. Among the strains producing the most pigment, MCIS1801, having insertions in the *fliCS* region, had the highest total carotenoid titer (~ 2.0 mg/g of DCW). MCIS1801 was generated via insertion of transposon Tn334, whereas the other strains containing transposon insertions in the *fliCS* regions, MCIS2201 and MCIS2203, were derived from insertion of the Tn341 transposon. Transposon Tn334 was responsible for all three transposon insertions detected in the *ccp-3* region. MCIS1701 and MCIS1804 contained transposon insertions in the *ccp-3* gene, whereas MCIS1702 had the carotenoid transposon inserted in the hypothetical open reading frame (mst1341) located upstream of the *ccp-3* gene. Strains MCIS1701, MCIS1804, and MCIS1702 had total carotenoid titers of ~ 1.7 , 1.1, and 1.9 mg/g of DCW, respectively (Table 2).

Two transposon insertions were detected in the gene located upstream of *hsdM* (mst1132) and the gene located upstream of *cysH* (mst2848). The Tn377 and Tn334 transposons were inserted once into each gene. *Methylobacter* strains MCIS2602, containing the Tn377 transposon, and MCIS1807, containing the Tn334 transposon, had total carotenoid titers that were ~ 0.5 and 1.3 mg/g of DCW, respectively. Insertion of the Tn377 and Tn334 transposons into the mst2848 region resulted in strains MCIS2601 and MCIS1703, which had total carotenoid titers that were ~ 0.6 and 1.7 mg/g of DCW, respectively.

DISCUSSION

The identification of chromosomal locations within the *Methylobacter* sp. strain 16a genome that support high-level carotenoid gene expression significantly improved our ability to metabolically engineer the obligate methanotroph for enhanced production of C_{40} carotenoids, specifically, astaxanthin. Five chromosomal regions that support elevated C_{40} carotenoid gene expression were identified in a genetic screen by random transposon mutagenesis. Transposable elements based on the Tn5 transposon were constructed to contain promoterless carotenoid gene clusters between the transposon ends. The carotenoid genes carried by the transposons were sufficient for the production of canthaxanthin, astaxanthin, and other carotenoid intermediates. By screening for colonies having the darkest hues and identifying the transposon insertion site, we discovered that regions of the *Methylobacter* chromosome involved in cell motility and assessment of the cellular redox state, as well as an unknown region located upstream of DNA methylation genes, enabled high-level carotenoid gene expression. By taking a random approach to identifying chromosomal locations that sustain enhanced carotenoid production, we have identified chromosomal regions that would not have been predicted by a rational approach.

Interestingly, three of the five regions (*ccp-3*, *cysH*, and *nirS*) contain genes that are involved in detoxification or serve as physiological reductants of oxygen (8). Considering that the *Methylobacter* sp. strain used in this study is an environmental isolate, it is reasonable that the expression of the *fliS* and *fliC* genes, which are involved in cell motility, is relatively active (11). This strain of *Methylobacter* has been cultivated in the laboratory for only a few years. Hence, it is likely that *Methylo-*

monas living in the environment would require the ability to move to locations of C-1 carbon abundance and other nutrients that are necessary for cell growth. We found that MCIS1801, which contains a Tn334 insertion within *fliS*, had the highest total carotenoid titer (~ 2.0 mg/g of DCW) of all of the strains evaluated. This suggests that some feature of the *fliCS* region, i.e., gene expression, stability of mRNA transcripts, or translation of the carotenoid mRNAs, is greatly improved in this strain. Two other insertions in the *fliCS* region with a different transposon, Tn341, which contains genes required for the synthesis of canthaxanthin, were also identified. The total carotenoid levels for *Methylobacter* strains containing Tn341 insertions in the *fliCS* region were relatively high compared to Tn341 insertions at other locations. It was observed that the total carotenoid levels of Tn341 insertions were $\sim 50\%$ of the total carotenoid levels obtained by insertions into the same region but by a Tn334 transposon. This finding suggested that the performances of the various C_{40} carotenoid transposable elements are not equal. Nevertheless, it is a significant discovery that the *fliCS* region was identified multiple times in independent genetic screens, suggesting that the *fliCS* region is an ideal location for the integration of C_{40} carotenoid genes to obtain high chromosomal expression. However, it should be noted that the stability of carotenoid production in strains containing transposon insertions in the *fliCS* region is not equal. Some strains that produced high levels of C_{40} carotenoids were very unstable, and the instability of these strains appeared specific to the insertion site and not specific to the particular gene that was disrupted. For example, when we compared strains MCIS1801 and MCIS2201, both containing insertions within *fliS*, for stability of carotenoid production, we found that they were drastically different. Strain MCIS1801 was shown to have unstable carotenoid production in bottle studies and in 2-liter continuous fermentations (unpublished results). In contrast, the other strains containing insertions in the *fliCS* region had very stable carotenoid production.

The *ccp-3* region of the *Methylobacter* chromosome was also repeatedly identified in a screen for carotenoid mutations that led to increased overall carotenoid titers. In this case, three strains were isolated from a single Tn334 transposon library. We suspect that the promoter responsible for driving expression is located upstream of *ccp-3*. One hypothesis that explains the high-level expression of the gene upstream of *ccp-3* compared to the other insertions within this region is the close proximity of the Tn334 insertion site within MCIS1702 to the promoter region of the gene upstream of *ccp-3*. Interestingly, however, comparison of the total carotenoid levels of two strains (MCIS1804 and MCIS1701) that contain insertions within *ccp-3* revealed different outcomes. MCIS1701, which had the Tn334 transposon inserted near the carboxyl terminus of Ccp-3, produced $\sim 35\%$ more total carotenoids. Although *ccp-3* is not essential under all growth conditions, it is possible that expression of cytochrome *c* peroxidase is advantageous to cell viability. Hence, the disruption of *ccp-3* near the 5' end of the gene would result in a truncated nonfunctional enzyme whereas insertion of Tn334 near the 3' end of *ccp-3* could result in a truncated protein that still contains functional domains. It is worth noting that, in addition to identifying chromosomal locations that support high-level carotenoid production, random transposition of Tn334 can also disrupt the

function of some genes upon insertion, offering a secondary effect of the insertion, including strain instability. When cells are cultivated under large-scale fermentation conditions, there is a requirement that the production strain be stable for many generations; therefore, all highly productive carotenoid-producing strains were also evaluated for strain stability by serial passage of the cultures ~30 times (data not shown).

Variability in the performance of the different carotenoid transposons was also observed. Note that Tn377 has consistently poorer performance compared to the Tn334 transposon, even when the transposons were inserted into similar locations within the *Methylomonas* genome. It has also been observed that carotenoid production is also influenced by gene order, i.e., the order in which the carotenoid genes are expressed (unpublished results).

The strain construction approaches described in this report greatly enhanced the metabolic engineering of *Methylomonas* strains for the production of several C₄₀ carotenoids. It has been shown that in vivo transposition with promoterless carotenoid transposons can be used to identify the chromosomal regions that support high-level and stable C₄₀ carotenoid gene expression. In addition to alleviating the burden of plasmid expression of the carotenoid genes, there are no antibiotic resistance genes present in these carotenoid-producing strains because of the use of the markerless chromosomal integration system described in this report. The construction of antibiotic resistance-free strains is ideal for large-scale fermentations, as well as for some applications in obtaining regulatory approvals. Carotenoids such as lycopene, β -carotene, canthaxanthin, and astaxanthin have been found to be very valuable in food and feed applications. Furthermore, the approach of using promoterless transposons containing genes of interest when metabolically engineering production strains could prove advantageous for producing many different compounds in a variety of microorganisms.

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REFERENCES

1. Antoine, R., and C. Loch. 1992. Isolation and molecular characterization of a novel broad-host range plasmids from *Bordetella bronchiseptica* with sequence similarities to plasmids from gram-positive organisms. *Mol. Microbiol.* **6**:1785–1799.
2. Chaffin, D. O., and C. E. Rubens. 1998. Blue/white screening for recombinant plasmids in gram-positive bacteria by interruption of alkaline phosphatase gene (*phoZ*) expression. *Gene* **219**:91–99.
3. Gat, O., I. Inbar, R. Aloni-Grinstein, E. Zahavy, C. Kronman, I. Mendelson, S. Cohen, B. Velan, and A. Shafferman. 2003. Use of promoter trap system in *Bacillus anthracis* and *Bacillus subtilis* for the development of recombinant protective antigen-based vaccines. *Infect. Immun.* **71**:801–813.
4. Herrero, M., V. DeLorenzo, and K. N. Timmis. 1990. Transposon vectors containing nonantibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
5. Higrera-Ciapara, L., L. Felix-Valenzuela, and F. M. Goycoolea. 2006. Astaxanthin: a review of its chemistry and applications. *Crit. Rev. Food Sci. Nutr.* **46**:185–196.
6. Hols, P., T. Rerain, D. Garmyn, N. Bernard, and J. Delcour. 1994. Use of homologous expression-secretion signals and vector-free stable chromosomal integration in engineering of *Lactobacillus plantarum* for α -amylase and levanase expression. *Appl. Environ. Microbiol.* **60**:1401–1413.
7. Israelsen, H. S., M. Madsen, A. Vrang, E. B. Hansen, and E. Johansen. 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-*lacZ* integrants with the new promoter probe vector, pAK80. *Appl. Environ. Microbiol.* **61**:2540–2547.
8. Karlyshev, A. V., M. J. Pallen, and B. W. Wren. 2000. Single-primer PCR procedure for rapid identification of transposon insertion sites. *BioTechniques* **28**:1078, 1080, 1082.
9. Machowski, E. E., R. A. McAdam, K. M. Derbyshire, and V. Mizrahi. 2000. Construction and application of mycobacterial reporter transposons. *Gene* **253**:67–75.
10. MacNab, R. M. 1996. Flagella and motility, p. 123–145. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, D. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
11. Majander, K., T. K. Korhonen, and B. Westerlund-Wikstrom. 2005. Simultaneous display of multiple foreign peptides in the FliD capping and FliC filament proteins of the *Escherichia coli* flagellum. *Appl. Environ. Microbiol.* **71**:4263–4268.
12. Matthyse, A. G., S. Stretton, C. Dandie, N. C. McClure, and A. E. Goodman. 1996. Construction of GFP vectors for use in gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiol. Lett.* **145**:87–94.
13. Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
14. Misawa, N., and H. Shimada. 1998. Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. *J. Biotechnol.* **59**:169–181.
15. Misawa, N., M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima. 1990. Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J. Bacteriol.* **172**:6704–6712.
16. Miura, Y., K. Kondo, T. Saito, H. Shimada, P. D. Fraser, and N. Misawa. 1998. Production of the carotenoid lycopene, β -carotene, and astaxanthin in the food yeast *Candida utilis*. *Appl. Environ. Microbiol.* **64**:1226–1229.
17. Miura, Y., K. Kondo, H. Shimada, T. Saito, K. Nakamura, and N. Misawa. 1998. Production of lycopene by the food yeast *Candida utilis* that does not naturally synthesize carotenoid. *Biotechnol. Bioeng.* **58**:306–308.
18. Nagarajan, V., H. Albertson, M. Chen, and J. Ribbe. 1992. Modular expression and secretion vectors for *Bacillus subtilis*. *Gene* **114**:121–126.
19. Sedkova, N., L. Tao, P. E. Rouviere, and Q. Cheng. 2005. Diversity of carotenoid synthesis gene cluster from environmental *Enterobacteriaceae* strains. *Appl. Environ. Microbiol.* **71**:8141–8146.
20. Shimada, H., K. Kondo, P. D. Fraser, Y. Miura, T. Saito, and N. Misawa. 1998. Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. *Appl. Environ. Microbiol.* **64**:2676–2680.
21. Simon, R., J. Quandt, and W. Klipp. 1989. New derivatives of transposon Tn5 for mobilization of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. *Gene* **80**:161–169.
22. Tao, L., A. Schenzle, J. M. Odom, and Q. Cheng. 2005. Novel carotenoid oxidase involved in biosynthesis of 4,4'-diapolyconene dialdehyde. *Appl. Environ. Microbiol.* **71**:3294–3301.
23. Winson, M. K., S. Swift, P. J. Hill, C. M. Sims, G. Griesmayr, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart. 1998. Engineering the *luxCDABE* genes from *Photobacterium luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn5 constructs. *FEMS Microbiol. Lett.* **163**:193–202.