

Genetic Characterization of the Carotenoid Biosynthetic Pathway in *Methylobacterium extorquens* AM1 and Isolation of a Colorless Mutant

Stephen J. Van Dien,¹ Christopher J. Marx,^{2†} Brooke N. O'Brien,² and Mary E. Lidstrom^{1,2*}

Department of Chemical Engineering¹ and Department of Microbiology,² University of Washington, Seattle, Washington 98195-2180

Received 30 June 2003/Accepted 22 September 2003

Genomic searches were used to reconstruct the putative carotenoid biosynthesis pathway in the pink-pigmented facultative methylotroph *Methylobacterium extorquens* AM1. Four genes for putative phytoene desaturases were identified. A colorless mutant was obtained by transposon mutagenesis, and the insertion was shown to be in one of the putative phytoene desaturase genes. Mutations in the other three did not affect color. The tetracycline marker was removed from the original transposon mutant, resulting in a pigment-free strain with wild-type growth properties useful as a tool for future experiments.

Methylotrophic bacteria are capable of growth using C₁ compounds such as methanol as their only carbon and energy source, and therefore they could potentially serve as biocatalysts for the conversion of methanol to useful products. Methanol is a good candidate for an alternative feedstock, as it is abundant, soluble, and low in cost. The pink-pigmented facultative methylotroph *Methylobacterium extorquens* AM1 is known to generate a pink carotenoid (6, 11). Since carotenoids have a variety of industrial uses (4), the carotenoid biosynthesis pathway is a potential target for metabolic engineering of this bacterium. In this work, genomic reconstruction and mutagenesis were used to characterize the carotenoid biosynthetic pathway in *M. extorquens* AM1.

Reconstruction of the carotenoid biosynthetic pathway from the genome sequence. The biosynthetic pathway for carotenoids has been elucidated through extensive study of various plants and bacteria (3) and is outlined in Fig. 1. The first part of the pathway, common to all isoprenoids, is the production of isopentenyl pyrophosphate (IPP). In most bacteria, this occurs through a pathway that begins with glyceraldehyde-3-phosphate and proceeds through 1-deoxy-D-xylulose-5-phosphate (DXP), as shown in Fig. 1 (13). This is referred to as the mevalonate-independent pathway. Although the details of the conversion to IPP are not fully understood, some of the genes in these intermediate steps have recently been identified (3, 12). Protein sequences of the enzymes involved in the upper pathway were obtained from the GenBank (<http://www.ncbi.nlm.nih.gov>) database and used to search the *M. extorquens* AM1 partial genome sequence (<http://www.integratedgenomics.com/genomereleases.html#list6>). Putative gene function was further evaluated by a BLAST search of the corresponding translated sequence against the National Center for Biotech-

nology Information database (<http://www.ncbi.nlm.nih.gov/BLAST>). The results of this search are listed in Table 1. Genes potentially encoding all of the steps of the pathway were found, with the exception of the IPP delta isomerase (*idi* gene). Since the production of IPP is essential for all organisms, it is likely that this gene is either divergent or located in the unsequenced portion of the genome. The other pathway for IPP synthesis, uncommon in bacteria, proceeds through mevalonic acid as an intermediate (3). No genes predicted to encode enzymes of this pathway were identified in the *M. extorquens* AM1 genome sequence.

IPP and its isomer dimethylallyl pyrophosphate are condensed to various degrees to form a wide variety of isoprenoid compounds, some of which are essential for growth. The first step unique to carotenoid synthesis is the condensation of four such units to make geranylgeranyl diphosphate (GGPP) via the enzyme GGPP synthase. Two of these molecules are then condensed by phytoene synthase to form phytoene, a precursor of all carotenoids (3). Phytoene desaturases then introduce a variable number of desaturations to create molecular diversity. The *Rhodobacter capsulatus* phytoene desaturase introduces three desaturations to produce neurosporene, which is yellow, while phytoene desaturases from many other bacteria introduce four desaturations to produce the red compound lycopene (7). Beginning with these compounds, further diversity in carotenoids is introduced by modifications such as desaturation, methylation, and hydroxylation. Examples include β -carotene, zeaxanthin, and astaxanthin (3).

Identity searches were performed by using translated sequences of known *R. capsulatus* (1) and *Erwinia herbicola* (8, 15) genes to locate putative carotenoid biosynthetic genes in the *M. extorquens* AM1 partial genome sequence (Table 2), which were further evaluated as described above by using BLAST. The predicted carotenoid biosynthesis genes are not tightly clustered, as they are located on seven different contigs in the genome sequence, separated by intervening DNA. Genes predicted to encode all steps for the formation of the first colored carotenoid (e.g., lycopene) from IPP were identified in the genome. In addition, three candidates for genes

* Corresponding author. Mailing address: Department of Chemical Engineering, University of Washington, Box 352180, Seattle, WA 98195-2180. Phone: (206) 616-5282. Fax: (206) 616-5721. E-mail: lidstrom@u.washington.edu.

† Present address: 2215 Biomedical Physical Sciences, Michigan State University, East Lansing, MI 48824-4320.

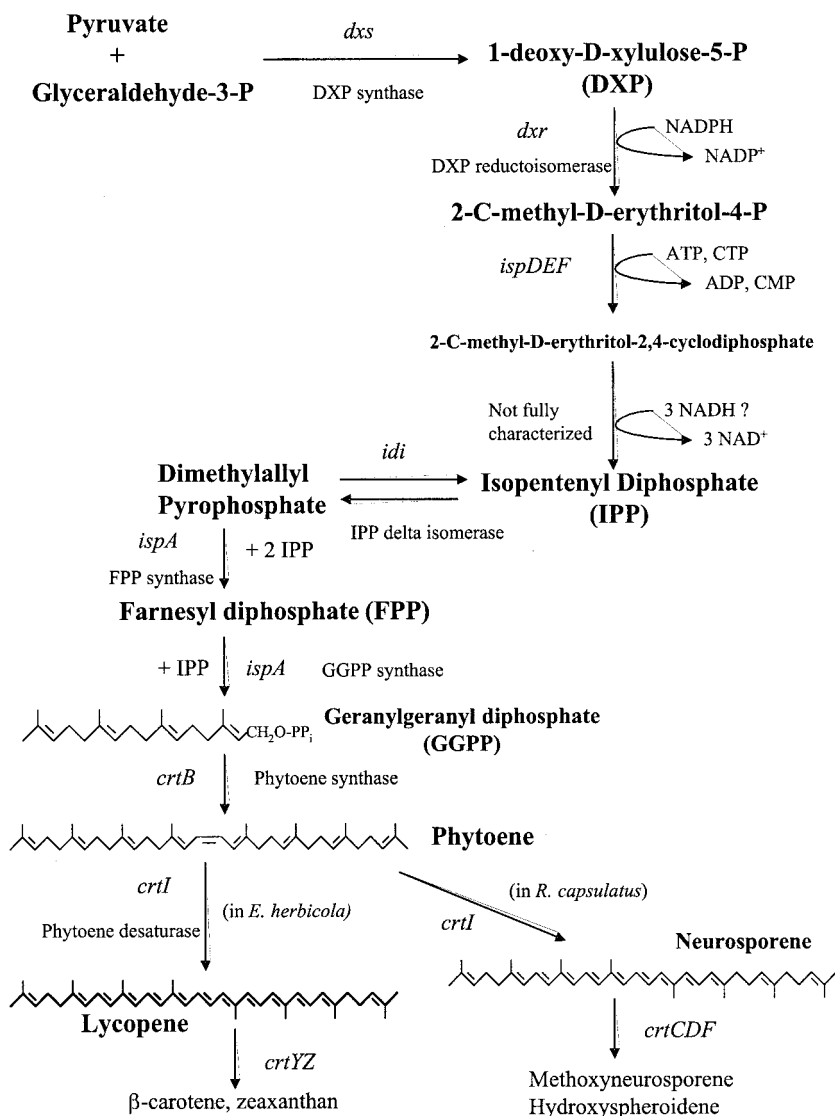


FIG. 1. Summary of bacterial carotenoid biosynthesis pathways and associated genes compiled from references 1, 3, 8, 12, and 13.

involved in further modification were found, with the highest identity to the hydroxyspheroidene pathway of *R. sphaeroides* (1). No candidates for the *Erwinia* β -carotene-zeaxanthan pathway (8) were found. Multiple candidates were identified for some carotenoid biosynthesis genes: two genes for phytoene synthase (*crtB*) and four for phytoene desaturase (*crtI*).

Isolation and genetic characterization of a colorless mutant of *M. extorquens* AM1 and generation of unmarked strain

AM1-W. Transposon mutagenesis using the mini-Tn5 derivative IS*phoA/hah*-Tc (5) was performed as previously described (10) in order to determine whether a single insertion mutation could result in loss of carotenoid production. Three white transposon mutants were isolated out of approximately 7,000 screened visually. The chromosomal site of insertion in each of these strains was determined as previously described (10). The sequences obtained were all mapped to the same open reading

TABLE 1. Summary of putative genes for the non-mevalonate pathway for IPP production in *M. extorquens* AM1^a

Gene(s)	Protein in GenBank database with lowest E value	BLASTP E value	% Identity	Accession no.
<i>dxp</i>	<i>R. capsulatus</i> DXP synthase	<1e-199	56	P26242 DXS_RHOCA
<i>dxs</i>	<i>Zymomonas mobilis</i> DXP reductoisomerase	2e-70	44	Q9X5F2 DXR_ZYMMO
<i>ispD-2ispF</i>	<i>R. capsulatus</i> IspD-IspF bifunctional protein	6e-47	44	Q08113 ISDF_RHOCA
<i>ispE</i>	<i>Z. mobilis</i> IspE	1e-28	35	Q9X3W5 ISPE_ZYMMO
<i>idi</i>	IPP delta isomerase gene not found			

^a Enzymes used for functional assignments are those found by a BLAST search of the translated gene sequence yielding the lowest E value.

TABLE 2. Summary of putative carotenoid biosynthesis genes found in the *M. extorquens* AM1 genome sequence^a

Gene(s)	Protein in GenBank with lowest E value	BLASTP E value	% Identity	Accession no.
<i>crtB</i>	<i>R. capsulatus</i> phytoene synthase	1e-50	46	P17056 CRTB_RHOCA
<i>orf1</i>	<i>R. capsulatus</i> phytoene desaturase (dehydrogenase)	1e-121	47	P17054 CRTI_RHOCA
<i>zdsA</i>	<i>Anabaena</i> sp. zeta-carotene desaturase	3e-66	32	D26095
<i>crtZ</i>	<i>R. capsulatus</i> phytoene desaturase	2e-61	31	P17054/CRTI_RHOCA
Gap of 4 putative ORFs				
<i>crtI</i>	<i>Erwinia herbicola</i> phytoene desaturase	2e-50	31	AAA64981
<i>crtB</i>	<i>Targetus erecta</i> (marigold) phytoene synthase	5e-22	37	AAM45379
<i>orf2</i>	<i>Streptomyces coelicolor</i> putative phytoene desaturase	0.003	24	NP_630834
<i>crtD</i> (incomplete sequence)	<i>Rubrivax gelatinosus</i> methoxyneurosporene desaturase	3e-70	44	AAC44798
<i>crtE</i>	<i>R. sphaeroides</i> GGPP synthase	7e-51	42	P54976 CRTE_RHODSH
<i>crtF</i>	<i>R. sphaeroides</i> methoxyneurosporene methyltransferase	4e-49	39	T50751
<i>ispD-ispF</i>	IspD-IspF (see Table 1)			
<i>ddsA</i>	<i>Paracoccus zeaxanthinifaciens</i> decaprenyl diphosphate synthase (quinone biosynthesis)	4e-84	54	CAD24417
<i>ispA</i>	<i>R. sphaeroides</i> FPP synthase	6e-43	46	BAA96457
<i>crtC</i> (incomplete sequence)	<i>R. sphaeroides</i> hydroxyneurosporene synthase	8e-12	42	QO167055 CRTC_RHOSH

^a Abbreviations are given in Fig. 1. Genes are grouped as they are clustered in the genome, with empty lines separating clusters. Enzymes used for the functional assignment are those found by a BLAST search of the translated gene sequence yielding the lowest E value.

frame (ORF) in the *M. extorquens* AM1 genome sequence: one of the four genes predicted to encode phytoene desaturase (phytoene desaturase). This gene is shown in bold in Table 2 and will be referred to as *crtI*.

For future genetic screening of this strain with colorimetric substrates, it would be useful to have a colorless, unmarked mutant. In order to generate such a pigment-free mutant, the tetracycline resistance gene of *crtI::ISphoA/hah-Tc* mutant S234-13 was removed by using *cre* expression plasmid pCM158 (9). The resulting unmarked, white mutant of *M. extorquens* AM1, AM1-W, was confirmed by analytical PCR assay. Under all standard laboratory conditions, growth of AM1-W was indistinguishable from that of the pigmented wild-type strain (data not shown).

To verify that *crtI* is responsible for the loss of color in the mutants, as opposed to a polar effect, gene complementation was performed. A 1.5-kb region containing *crtI* was amplified by PCR, cloned directly into pCR2.1 (Invitrogen), and subcloned as a *Bam*HI-*Eco*RI fragment into pCM66 and pCM160 (10) to produce pCAR12 and pCAR14. The resulting plasmids contain *crtI* expressed from *Escherichia coli* *P*_{lac} or *M. extorquens* AM1 *P*_{msaF}, respectively. Both plasmids were introduced by conjugation with *E. coli* S17-1 (16) into AM1-W, resulting in pink colonies. In contrast, colonies of AM1-W containing either pCM66 or pCM160 remained white. These results demonstrate that the *M. extorquens crtI* gene is essential for formation of the pink pigment and confirm previous suggestions (6, 11) that the pigment was due to the presence of a carotenoid.

Surprisingly, *crtI* was not the most likely candidate to encode an active phytoene desaturase, on the basis of the information in Table 2. The gene labeled *orf1* is located immediately downstream of a putative phytoene synthase gene and has greater identity to a known phytoene desaturase. In contrast, *crtI* is surrounded by putative phospholipid and cell wall biosynthesis genes and a GMP synthase. Deletion mutants with changes in two of the remaining phytoene desaturase gene candidates, *orf2* and *orf3*, were prepared by using PCR products of these ORFs and the allelic exchange vector pCM184 (9). These mutants were confirmed to be double crossovers by PCR analysis, indicating that they have null mutations in the corresponding gene. Both mutants remained pink, suggesting that these genes do not play a major role in the synthesis of colored carotenoids in *M. extorquens* AM1.

Isolation of carotenoid from wild-type *M. extorquens* AM1. Carotenoids were extracted from *M. extorquens* AM1 by using a modification of the procedure commonly used for photosynthetic bacteria (17). Cell pellets obtained from approximately 100 ml of cell culture grown in mineral salts medium (2) containing 125 mM methanol were resuspended in 1 ml of methanol at 65°C and vortexed for 1 min. A 0.4-ml volume of water and 0.3 ml of chloroform were added, and the sample was vortexed for an additional 2 min. The bottom organic layer, containing the carotenoid, was extracted into clean tubes. A 1-ml volume of methanol and 0.4 ml of water were added, and the organic layer was extracted again. The extract from wild-type cells was pink, whereas that from mutant strain AM1-W was colorless. A 2-ml volume of acetone was added to the

extract (about 0.15 ml) in order to remove phospholipids and glycolipids (14), and the sample was placed at -20°C overnight. The sample was centrifuged, and the supernatant was evaporated to dryness and redissolved in 0.1 ml of chloroform. The visible absorption spectrum of the sample, with maxima at 480, 510, and 540 nm, matched that of the extract from a related pink-pigmented methylotroph, previously referred to as *Protaminobacter ruber* (14). Furthermore, as observed with the carotenoid from *P. ruber* (14), a 9-nm red shift in the absorption spectra was observed following hydrolysis of the carotenoid in 0.2 M HCl-methanol. These results suggest that the pink carotenoid in *M. extorquens* AM1 is similar to that in *P. ruber*, which has been shown to be a carotenoid with an attached sugar moiety that is lost during hydrolysis. The ^1H nuclear magnetic resonance spectrum of the *P. ruber* extract indicated the presence of a carbonyl group and about 13 conjugated double bonds, and thin-layer chromatography data suggested that the carotenoid has a bacterioruberin- or oscillaxanthin-like structure (14).

Nucleotide sequence accession number. The sequence of *M. extorquens* AM1 *crtI* has been deposited in the GenBank database (accession number AY331188).

This work was supported by a grant from the National Institutes of Health (GM58933).

REFERENCES

1. Armstrong, G. A., M. Alberti, F. Leach, and J. E. Hearst. 1989. Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. *Mol. Gen. Genet.* **216**:254–268.
2. Attwood, M. M., and W. Harder. 1972. A rapid and specific enrichment procedure for *Hyphomicrobium* spp. *Antonie Van Leeuwenhoek* **38**:369–377.
3. Barkovich, R., and J. C. Liao. 2001. Metabolic engineering of isoprenoids. *Met. Eng.* **3**:27–39.
4. Bauernfeind, J. C. 1981. Carotenoids as colorants and vitamin A precursors: technological and nutritional applications. Academic Press, Inc., New York, N.Y.
5. D'Argenio, D. A., L. A. Gallagher, C. A. Berg, and C. Manoil. 2001. *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J. Bacteriol.* **183**:1466–1471.
6. Downs, J., and D. E. F. Harrison. 1974. Studies on the production of pink pigment in *Pseudomonas extorquens* NCIB 9399 growing in continuous culture. *J. Appl. Bacteriol.* **37**:65–74.
7. Garcia-Asua, G., H. P. Lang, R. J. Cogdell, and C. N. Hunter. 1998. Carotenoid diversity: a modular role for the phytoene desaturase step. *Trends Plant Sci.* **3**:445–449.
8. Hundle, B., M. Alberti, V. Nievelstein, P. Beyer, H. Kleinig, G. A. Armstrong, D. H. Burke, and J. E. Hearst. 1994. Functional assignment of *Erwinia herbicola* Eho10 carotenoid genes expressed in *Escherichia coli*. *Mol. Gen. Genet.* **245**:406–416.
9. Marx, C. J., and M. E. Lidstrom. 2002. Broad-host-range *cre-lox* system for antibiotic marker recycling in gram-negative bacteria. *BioTechniques* **33**:1062–1067.
10. Marx, C. J., B. N. O'Brien, J. Breezee, and M. E. Lidstrom. 2003. Novel methylotrophy genes of *Methylobacterium extorquens* AM1 identified using transposon mutagenesis including a putative dihydromethanopterin reductase. *J. Bacteriol.* **185**:669–673.
11. Peel, D., and J. R. Quayle. 1961. Microbial growth on C_1 compounds. 1. Isolation and characterization of *Pseudomonas* AM1. *Biochem. J.* **81**:465–469.
12. Rohdich, F., J. Wungsintaweeikul, H. Luttgen, M. Fisher, W. Eisenreich, C. A. Schuhr, M. Fellermeier, N. Schramek, M. H. Zenk, and A. Bacher. 2000. Biosynthesis of terpenoids: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase from tomato. *Proc. Natl. Acad. Sci. USA* **97**:8251–8256.
13. Rohmer, M. 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae, and higher plants. *Nat. Prod. Rep.* **16**:565–574.
14. Sato, K., T. Mizutani, M. Hiraoka, and S. Shimizu. 1982. Carotenoid containing sugar moiety from a facultative methylotroph, *Protaminobacter ruber*. *J. Ferment. Technol.* **60**:111–115.
15. Schnurr, G., A. Schmidt, and G. Sandmann. 1991. Mapping of a carotenogenic gene cluster from *Erwinia herbicola* and functional identification of six genes. *FEMS Microbiol. Lett.* **62**:157–161.
16. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1**:784–791.
17. Takaichi, S., and K. Shimada. 1992. Characterization of carotenoids in photosynthetic bacteria. *Methods Enzymol.* **213**:374–385.