

A C₃₅ Carotenoid Biosynthetic Pathway

Daisuke Umeno* and Frances H. Arnold*

Division of Chemistry and Chemical Engineering, California Institute of Technology,
Pasadena, California 91125

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Upon coexpression with *Erwinia* geranylgeranyl diphosphate (GGDP) synthase in *Escherichia coli*, C₃₀ carotenoid synthase CrtM from *Staphylococcus aureus* produces novel carotenoids with the asymmetrical C₃₅ backbone. The products of condensation of farnesyldiphosphate and GDP, C₃₅ structures comprise 40 to 60% of total carotenoid accumulated. Carotene desaturases and carotene cyclases from C₄₀ or C₃₀ pathways accepted and converted the C₃₅ substrate, thus creating a C₃₅ carotenoid biosynthetic pathway in *E. coli*. Directed evolution to modulate desaturase step number, together with combinatorial expression of the desaturase variants with lycopene cyclases, allowed us to produce at least 10 compounds not previously described. This result highlights the plastic and expansible nature of carotenoid pathways and illustrates how combinatorial biosynthesis coupled with directed evolution can rapidly access diverse chemical structures.

Impressively diverse and rich in biological activity, natural secondary metabolites comprise but a fraction of the chemicals that could be made by biological systems. Some new metabolites can be accessed by coexpressing biosynthetic genes from different sources in a recombinant host (combinatorial biosynthesis [22–25, 28]). Alternatively, the new pathways can also be evolved, or bred, in the laboratory; here, new biosynthetic functions are created within the context of a pathway by random mutagenesis and recombination of biosynthetic genes coupled with screening for the products of newly emerging branches (26, 27). For this strategy to be useful, it is important to understand how natural secondary metabolic pathways are structured, how they can rapidly explore new chemical structures, and, most importantly, how we can use evolutionary algorithms to accelerate this search.

Secondary metabolic pathways appear to be able to explore new chemical structures at minimal cost (7). Key to promoting facile exploration is the extensive use of promiscuous enzymes that can accept a variety of substrates. Thus, once a new compound is produced, as a result of either enzyme recruitment or mutation in an existing enzyme, it is further metabolized by downstream modifying enzymes, thereby allowing a series of novel compounds to emerge. Such “hidden” branches have been found in several natural pathways (5, 9, 10, 15). In breeding pathways in the laboratory, we can exploit this feature to generate new molecular diversity.

Carotenoids are natural pigments that play various biological roles (3, 14, 18, 20). At least 700 carotenoids have been characterized (12) from the two known carotenoid biosynthetic pathways. Most widely distributed is the C₄₀ pathway, which is shared by thousands of plant and microbial species. In this pathway, two molecules of geranylgeranyldiphosphate (GGDP) (C₂₀PP) are condensed to form phytoene (Fig. 1, compound 3). The second, C₃₀ pathway is known in only a few bacterial

species, such as those of *Staphylococcus* and *Heliobacterium* (29, 31, 32). Here, two molecules of farnesyldiphosphate (FDP) (C₁₅PP) undergo condensation to form 4,4'-diapophytoene (dehydrosqualene) (Fig. 1, compound 1). Homo (>C₄₀-) and apo (<C₄₀-) carotenoids are also known; they derive from C₄₀ carotenoid precursors (1, 2, 16, 17). Most natural carotenoid diversity arises from differences in types and levels of desaturation and other modifications of the C₄₀ backbone. Why nature has chosen these two pathways and not others is not known. We are testing whether novel pathways for non-C₃₀ and non-C₄₀ carotenoids can be created and how easily such new pathways generate molecular diversity. Here we report the construction of a C₃₅ carotenoid pathway in *Escherichia coli*. This pathway emerged when the C₃₀ carotene synthase CrtM from *Staphylococcus aureus* was supplied with the natural substrate of the C₄₀ synthase, GGDP. Other carotenoid-synthesizing enzymes, specifically, carotene cyclases and desaturases from C₄₀ and C₃₀ pathways, were found to be functional on the C₃₅ backbone, and thus a C₃₅ carotenoid pathway was quickly established. Directed evolution of a carotene desaturase yielded variants with altered step number and increased the variety of C₃₅ carotenoids that could be produced by the new pathway.

MATERIALS AND METHODS

Materials. *crtE* (GGDP synthase), *crtB* (phytoene synthase), *crtI* (phytoene desaturase), and *crtY* (β-end lycopene cyclase) from *Erwinia uredovora* were obtained by genomic PCR as described previously (27). *crtM* (diapophytoene synthase) and *crtN* (diapophytoene desaturase) were PCR cloned from genomic DNA of *S. aureus* (ATCC 35556). Lettuce *dy4* (ε-end lycopene cyclase) (6) was kindly provided by Francis X. Cunningham, Jr. (University of Maryland). AmpliTaq polymerase (Perkin-Elmer, Boston, Mass.) was used for mutagenic PCR, while Vent polymerase (New England Biolabs, Beverly, Mass.) was used for cloning PCR. All chemicals and reagents were of the highest available grade.

Plasmid construction. To express carotenoid biosynthetic enzymes, we used the *lac* promoter system devoid of operator sequence, as described previously (27). Instead of providing promoters for each pathway component, however, we placed multiple genes under the control of a single *lac* promoter. Each open reading frame, following a Shine-Dalgarno sequence (in bold) and a spacer (AGGAGGATTACAAA), was PCR cloned into the plasmid to form artificial operons. Genes in plasmids and operons are always listed in transcriptional order. Carotene synthases (*crtM* and *crtB*) are flanked by *XbaI*-*XbaI* sites, car-

* Corresponding author. Mailing address: Division of Chemistry and Chemical Engineering, California Institute of Technology, 210-41 1200 E. California Blvd., Pasadena, CA 91125. Phone: (626) 395-4162. Fax: (626) 568-8743. E-mail for D. Umeno: umeno@chem.caltech.edu. E-mail for F. H. Arnold: frances@chem.caltech.edu.

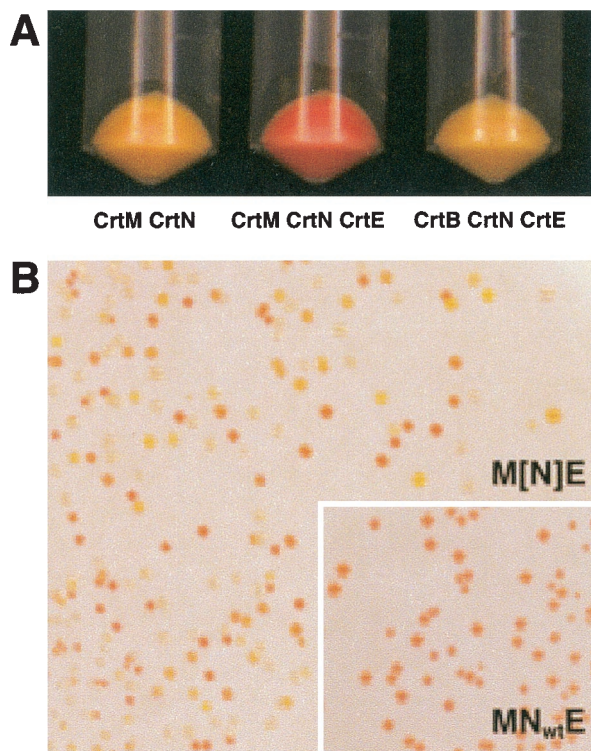


FIG. 2. (A) Cell pellet of XL1 cells harboring pUC-*crtM-crtN*, pUC-*crtM-crtN-crtE*, and pUC-*crtB-crtN-crtE*. (B) Typical view of agar plates of XL1 cells transformed with the pUC-*crtM*-[*crtN*]-*crtE* library.

RESULTS

C₃₀ carotenoid enzymes shift their pigment production in the presence of GGDP. For the production of C₃₀ carotenoids, we subcloned *crtM* (dehydrosqualene synthase) and *crtN* (dehydrosqualene desaturase), both from *S. aureus* (35), into a pUC19 derivative (27) to generate plasmid pUC-*crtM-crtN*. *E. coli* XL1 transformed with pUC-*crtM-crtN* shows a typical yellow color due to C₃₀ carotenoid production. In contrast, when XL1 cells were transformed with pUC-*crtM-crtN-crtE*, where *crtE* (GGDP synthase) from *E. uredoavora* was additionally expressed, they exhibited an intense red color (Fig. 2A). The spectrum of the acetone extract has a shoulder at 525 nm, indicating the presence of a polyene containing 13 conjugated double bonds. Because the C₃₀ backbone accommodates only 11 conjugated double bonds, it appeared that the cells were producing a >C₃₀ carotenoid. *CrtN* was previously shown to be functional to some extent on C₄₀ carotenoids, but both in vitro and in vivo experiments showed that it is not more than a three-step desaturase in a C₄₀ pathway (21, 33). Indeed, XL1 cells harboring pUC-*crtB-crtN-crtE* (with the C₄₀ synthase) are yellow (Fig. 2A) and solely accumulate neurosporene. Therefore, the source of the red hue of XL1 cells transformed with pUC-*crtM-crtN-crtE* was believed to be a novel carotenoid with a non-C₃₀, non-C₄₀ backbone.

***CrtM* produces 4-diapophytoene in the presence of GGDP.** *E. coli* cells harboring pUC-*crtE-crtB* exclusively produced phytoene (C₄₀) (compound 3; M⁺ at *m/e* = 544.5) (Fig. 3C), while *E. coli* carrying pUC-*crtB* accumulated undetectable amounts of carotenoids (data not shown). Thus, *crtB* appears to be a

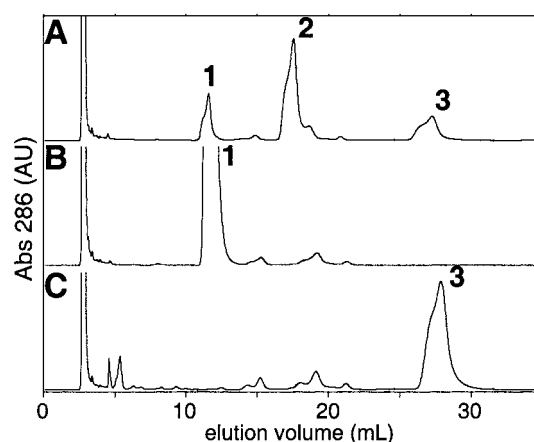


FIG. 3. HPLC analysis of carotenoid extract from HB101 cells carrying plasmids pUC-*crtM-crtE* (A), pUC-*crtM* (B), and pUC-*crtE-crtB* (C). Individual compounds are as follows: peak 1, 4,4'-diapophytoene; peak 2, 4-apophytoene; peak 3, phytoene. Elution conditions: ODS-2 column; 1 ml/min; acetonitrile-2-propanol (80:20). The detection wavelength was 286 nm.

specific C₄₀ synthase. *E. coli* cells transformed with pUC-*crtM* produced only diapophytoene (C₃₀) (compound 1; *m/e* = 408.5) (Fig. 3B). When *E. coli* was transformed with pUC-*crtM-crtE*, however, three carotenoids accumulated: in addition to phytoene (C₄₀) and 4,4'-diapophytoene (C₃₀), a novel phytoene-type carotenoid, 4-apophytoene (C₃₅) appeared (compound 2; *m/e* = 476.5) (Fig. 3A). We believe that this carotenoid is synthesized via heterocondensation of FDP (C₁₅) and GGDP (C₂₀) (C₁₅ + C₂₀ = C₃₅ [Fig. 1]) catalyzed by *CrtM*.

Among the *E. coli* strains tested, HB101 was the best producer of compound 2, both in its production level (220 to 350 nmol [100 to 170 μg] of C₃₅ carotenoid/g [dry mass] of cells) and in its proportion to the total carotenoids (~55%). BL21 and BL21Gold also showed similarly good C₃₅ production, while XL1, DH5α, XL10Gold, and TOP10 cells accumulated compound 2 at a slightly lower level (35 to 45 μg/g of cell; ca. 40% of total carotenoids). For those strains, no significant effects on cell growth were observed. On the other hand, JM109, JM101, and BL21(DE3) turned out to be very poor and unstable producers of C₃₅ carotenoids. This might partially explain why others who expressed the same combination of genes in JM101 (21) did not describe this compound. On the other hand, our production of compound 2 was reproducible and insensitive to changes in other parameters. Replacement of the pUC-derived expression vector (with a *ColE1* origin and ampicillin marker) with the pACYC184 derivative (with a p15A origin and chloramphenicol marker) gave virtually no change in the amount or composition of carotenoids produced. Likewise, carotenoid production by *E. coli* carrying pACYC-*crtM-crtE* and pUC-*crtN* or *E. coli* harboring pACYC-*crtN-crtE* and pUC-*crtM* was virtually the same (data not shown). This insensitivity to the expression system greatly facilitated the addition of carotenoid-modifying enzymes such as carotene cyclases. *E. coli* transformed with pUC-*crtM* produced only diapophytoene (C₃₀) (Fig. 3B, compound 1).

Upon coexpression of *CrtE* (GGDP synthase), production

of 4,4'-diapophytoene (the natural product) by CrtM dropped dramatically, to ca. 1/50 to 1/75 of that in its absence (ca. 1 mg/g of cell). This indicates that CrtE readily consumes FDP for GGDP production and that FDP for C₃₀ synthesis is severely limited. Based on this, it is assumed that C₃₅ production is the result of uptake of GGDP by CrtM when FDP is depleted.

Desaturation in the C₃₅ pathway. It is known that diapophytoene (C₃₀) desaturase (staphylococcal CrtN) is able to convert phytoene (C₄₀) to some extent. Likewise, phytoene desaturases can accept 4,4'-diapophytoene (21, 33). Thus, it was not surprising that the *S. aureus* CrtN (from a C₃₀ pathway) and *E. uredovora* CrtI (from a C₄₀ pathway) were functional in the C₃₅ pathway. Figure 4B and C show the HPLC analysis of the extracted pigments from XL1 transformed with pUC-*crtM-crtN-crtE* or pUC-*crtM-crtI-crtE*, respectively. Both cells accumulated two carotenoids, compounds 6 and 7, that were not found in cells without CrtE (Fig. 4A). Elution profiles, UV-visible spectra, and mass spectra (M^+ at $m/e = 466.5$ and 468.5 , respectively) confirmed that peak 6 is the fully conjugated C₃₅ carotenoid, 4-apo-3',4'-didehydrolycopene, while peak 7 corresponds to a C₃₅ carotenoid with 11 conjugated double bonds. Because C₃₅ carotenoids are asymmetric, there are two possible such C₃₅ structures, 4-apolycopene and 4-apo-3',4'-didehydro-7,8-dihydrolycopene (Fig. 1). At present, it is not known which is the actual structure corresponding to peak 7.

The apparent step number of CrtI was slightly greater than that of CrtN in the C₃₅ pathway, and XL1 cells carrying pUC-*crtM-crtI-crtE* accumulated compound 6 as the main product. The apparent *in vivo* activity of these desaturases in the C₃₅ pathway was high enough so that unconverted substrate (compound 2) and products with lower desaturation step numbers did not accumulate. Interestingly, C₃₀ desaturase CrtN showed a higher apparent step number (four or five steps) in the C₃₅ pathway than in its native C₃₀ pathway (three or four steps). Thus, cells with carotenoid biosynthetic enzymes CrtM and CrtN develop intense red color in the presence of GGDP, due to the production of highly desaturated C₃₅ carotenoids (Fig. 2A).

Cyclization of C₃₅ carotenoids. Most carotenoids in plants and microorganisms are in cyclic forms, and various carotene cyclases have been isolated. However, cyclization is known only for C₄₀ pathways. Several lycopene cyclases were reported to convert the 7,8-dihydro ψ end along the C₄₀ backbone in addition to their natural substrate, lycopene (ψ end) (30). We tested the function of two carotene cyclases, the β -end cyclase (CrtY) from *E. uredovora* and the ϵ -end cyclase from lettuce (Dy4), in the C₃₅ pathway.

When pUC-*crtY* was transformed into XL1 together with pAC-*crtM-crtN-crtE*, two new carotenoids (compounds 11 and 12) (Fig. 4G) were observed in small amounts. Their characteristic absorption spectra and molecular masses confirmed that compounds 11 (M^+ at $m/e = 468.4$) and 12 ($m/e = 470.4$) are the monocyclic β -end C₃₅ carotenoids shown in Fig. 1. Similarly, expression of pUC-*dy4* with pAC-*crtM-crtN-crtE* resulted in the production of two other carotenoids (compounds 13 and 14) (Fig. 4J). Absorption spectra and molecular masses suggested that compounds 13 ($m/e = 468.2$) and 14 ($m/e = 470.4$) are the monocyclic ϵ -end C₃₅ carotenoids shown in Fig. 1.

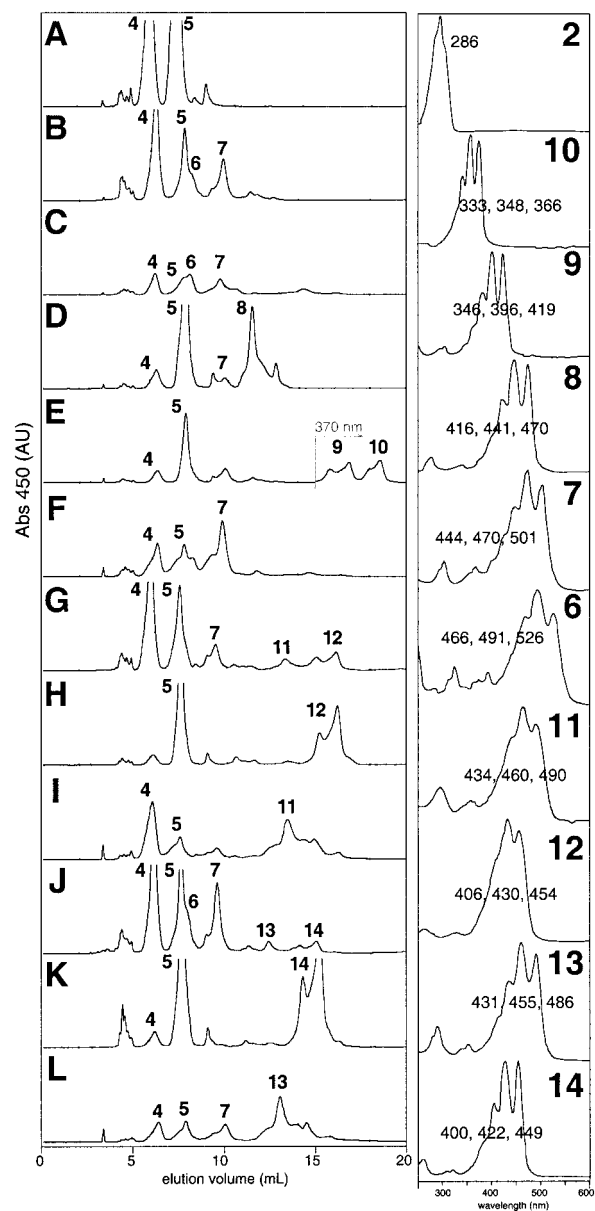


FIG. 4. HPLC profile of carotenoid extracts from XL1 cells carrying plasmids pUC-*crtM-crtN* (A), pUC-*crtM-crtN-crtE* (B), pUC-*crtM-crtI-crtE* (C), pAC-*crtM-N_{6A}-crtE* (D), pAC-*crtM-N_{10F}-crtE* (E), pAC-*crtM-I₁₃-crtE* (F), pUC-*crtM-crtI-crtE* and pUC-*crtY* (G), pAC-*crtM-N_{6A}-crtE* and pUC-*crtY* (H), pAC-*crtM-crtI₁₃-crtE* and pUC-*crtY* (I), pAC-*crtM-crtN-crtE* and pUC-*dy4* (J), pAC-*crtM-crtN_{6A}-crtE* and pUC-*dy4* (K), and pAC-*crtM-crtI₁₃-crtE* and pUC-*dy4* (L). Elution conditions: 1 ml/min; acetonitrile-2-propanol (85:15). The detection wavelength was 450 nm throughout the run, except for panel E, where 370 nm was used after 15 min. See Fig. 1 for putative structures for each carotenoid. The spectrum of each C₃₅ carotenoid (compounds 2 and 6 to 14) is given on the right.

Given the optical properties and molecular masses of the respective carotenoids, there are two possible structures for each of the four cyclic molecules 11 to 14 (structures **a** and **b** in Fig. 1). Although we do not have conclusive evidence, we believe that the cyclic carotenoids detected are of the **a** type rather than the **b** type. The **a**-type carotenoids are synthesized

by cyclase action on the C₂₀ half, whose ψ end is identical to that of lycopene, the natural substrate of CrtY and Dy4, while b-type pathways require cyclization on the shorter, unnatural (C₁₅) side. It has been noted that carotenoid-modifying enzymes in general recognize only a part of the substrate (4).

Modulating desaturase step number in the C₃₅ pathway.

Both CrtN and CrtI are four- or five-step desaturases in the C₃₅ pathway and therefore accumulate acyclic C₃₅ carotenoids with either 11 (for compound 6) or 13 (for compound 7) conjugated double bonds. To access C₃₅ carotenoids with five, seven, and nine conjugated double bonds, we used directed evolution to alter the step numbers of these desaturases in the pathway. Each desaturation step extends the chromophore by two double bonds, providing a large bathochromic shift and a basis for product-based color screening of desaturase variants with altered step number. This approach has been used to alter the step numbers of CrtI from *Erwinia* (27) and *Rhodobacter* (34), as well as that of CrtN from *Staphylococcus* (D. Umeno, unpublished data), in their respective natural pathways.

By using pUC-*crtM-crtN-crtE* as a template, a region covering the entire *crtN* reading frame was amplified by mutagenic PCR. The PCR product was digested and ligated into the desaturase site of pUC-*crtM-crtN-crtE*, resulting in the pUC-*crtM-[crtN]-crtE* plasmid libraries. Similarly, pUC-*crtM-[crtI]-crtE* libraries were created and screened for altered carotenoid production. When XL1 cells were transformed with the pUC-*crtM-[crtN]-crtE* libraries, we observed orange-red colonies similar to those expressing the wild-type CrtN, as well as orange, yellow, and virtually colorless colonies (Fig. 2B). About 100 yellow-to-white colonies were picked and inoculated in a 2-ml TB culture, and pigmentation was analyzed by the absorption spectrum of the pigment mixture extracted from each variant sample. Because each cell produces a mixture of C₃₀, C₄₀, and C₃₅ carotenoids, color changes could occur in various ways. In addition to an altered step number in the C₃₅ pathway, altered step numbers in other (C₃₀ or C₄₀) pathways, altered specificity or preference for backbone size, and possibly a change in the physical interaction with CrtM could generate the different phenotypes. Thus, promising variants were harvested in a larger-scale culture (50 ml of TB), and pigment composition was analyzed by HPLC.

From the variants analyzed, two CrtN variants (N_{6A} and N_{10F}) and a CrtI variant (I₁₃) were subcloned into the pACYC vector. While XL1 carrying pAC-*crtM-crtN_{wt}-crtE* accumulated a mixture of five- and four-step products (Fig. 4B), XL1 having pAC-*crtM-N_{6A}-crtE* accumulated three-step product 8 (M⁺ at $m/e = 470.4$) as a major product (Fig. 4D). On the other hand, transformation of XL1 with pUC-*crtM-N_{10F}-crtE* resulted in the accumulation of two-step (compound 9; $m/e = 472.4$) and one-step (compound 10; $m/e = 474.3$) products (Fig. 4E).

Improving cyclic C₃₅ carotenoid production. Both CrtY and Dy4 converted C₃₅ carotenoids in the presence of CrtM, CrtN, and CrtE (Fig. 4G and 4J). However, the proportion of cyclic C₃₅ carotenoids accumulated was rather low. This could simply mean that C₃₅ carotenoids are not good substrates for C₄₀ cyclases. Alternatively, it could be due to the suboptimal combination of cyclase and desaturase enzymes. The pathways to compounds 11 and 13 branch out from compound 7, while the paths to compounds 12 and 14 start from compound 8. In either case, the cyclization pathway must compete for substrate

with the desaturation pathway leading to compound 6. We proposed that the level of cyclic C₃₅ carotenoid could be enhanced by replacing wild-type CrtN with desaturase variants with appropriate step numbers that accumulate, but do not consume, the substrates for each planned pathway.

XL1 cells harboring pAC-*crtM-N_{6A}-crtE* accumulated compound 8 at high levels (Fig. 4D). When cyclases were coexpressed with this plasmid, compound 8 was efficiently converted into compounds 12 (by CrtY) (Fig. 4H) and 14 (by Dy4) (Fig. 4K). XL1 harboring pAC-*crtM-I₁₃-crtE* accumulates a high level of compound 7 (Fig. 4F), the direct precursor for compounds 11 and 13. Upon expression of cyclases CrtY and Dy4, compounds 11 (by CrtY) (Fig. 4I) and 13 (by Dy4) (Fig. 4L) were produced at high proportions. Thus, modulation of the precursor supply was sufficient to selectively produce each of four cyclic carotenoids. This context-dependent switching of the cyclization products demonstrates how readily these enzymes accept new substrates.

DISCUSSION

The C₃₅ pathway and evolution of the carotenoid pathways.

Many carotenoid-producing enzymes are promiscuous, accepting a range of substrates (9, 10, 21, 30). Carotene desaturases from C₃₀ and C₄₀ pathways can complement each other (21, 33). Nevertheless, C₄₀ carotenoids have not been isolated from C₃₀ carotenoid-synthesizing organisms (32), and C₃₀ carotenoids are not seen in C₄₀ organisms. This complete isolation of natural C₃₀ and C₄₀ carotenoid pathways occurs even though they are very similar, except in the size of their precursor molecules. Carotene synthases are the likely source of this specificity. It was shown that, unlike carotene desaturases, neither the C₃₀ nor the C₄₀ carotene synthase was functional in the other pathway (21, 33). Indeed, *Erwinia* CrtB produced C₄₀ carotenoid only, with not even a trace amount of C₃₅ or a smaller carotenoid, when expressed in *E. coli* also expressing CrtE, despite the availability of both GGDP and FDP (Fig. 3C). In the absence of GGDP, CrtB produced no carotenoids at all.

In this work, we have shown that *Staphylococcus* CrtM, a synthase from a C₃₀ pathway, synthesizes C₃₅ carotenoids in the presence of GGDP. CrtM can condense two FDP molecules; it can also accept one (or two) molecules of GGDP in the same reaction. In the absence of CrtE, however, CrtM produces C₃₀ carotenoids (4,4'-diapophytoene) exclusively (Fig. 3B). Thus, specificity of the C₃₀ pathway against the production of larger carotenoids is achieved by limiting the precursor pool (environment) and not by the synthase itself.

Asymmetry of carotenoid structures ensures greater molecular diversity in the C₃₅ pathway. Because C₃₅ carotenoids have asymmetrical backbone structures, each desaturation step along the backbone yields more than one product. Thus, acyclic C₃₅ carotenoids with 3, 5, 7, 9, 11, and 13 conjugated double bonds can take 1, 2, 3, 3, 2, and 1 (total of 12) possible structures, respectively (Fig. 1), while C₄₀ has 9 possible structures for 6 different chromophore sizes. Cyclization and other modifications of the backbone can further increase the number of possible C₃₅ carotenoids compared to symmetrical (natural) carotenoids. Thus, the C₃₅ pathway is inherently more complex

and explores a much larger “structure space” than the symmetrical C₃₀ and C₄₀ pathways.

Many enzymes in carotenoid pathways have been reported to have broad substrate specificity (9–11, 19, 21, 30), including the ability to act on carotenoids of different backbone size (21, 33). Many carotenoid-modifying enzymes are expected to be functional in the C₃₅ pathway, as were the four enzymes tested in this work (CrtN, CrtI, CrtY, and Dy4). We predict that the C₃₅ carotenoid pathway products can be metabolized by additional C₄₀ or C₃₀ enzymes, which will further expand the diversity of novel carotenoids that can be generated.

Matching of components enhances the discovery of novel chemicals. Due to their promiscuity, secondary metabolic enzymes can be expressed combinatorially to produce, in theory, a very large number of chemical structures. Garcia-Asua et al. observed the accumulation of a series of uncommon carotenoids in *Rhodobacter sphaeroides* when they replaced its natural three-step desaturase with a four-step counterpart from *Erwinia* (9, 10). Here we have demonstrated that addition of a single enzyme, GGDP synthase, creates a whole new pathway to C₃₅ carotenoids.

Most of the chemicals from this potentially rich source, however, are not accessible by simple assembly of natural biosynthetic enzymes. Often, this approach merely results in the production of unwanted compounds. For example, attempts to construct a pathway for astaxanthin production by expressing all of the required enzymes in a host organism resulted in astaxanthin formation as only a minor component of a complex mixture (11, 19). In another example, introduction of carotenoid biosynthetic enzymes from *Erwinia* (CrtB, CrtI, CrtY, and CrtZ) into a mutant of *Rhodobacter* with the desaturase deleted only restored the desaturation activity and failed to alter the organism’s carotenoid product range (13). In that case, the endogenous hydroxylation activity on neurosporene was so high that the activities introduced from *Erwinia* failed to compete for this intermediate. The additional knockout of neurosporene hydroxylase CrtC was necessary to make the pathway functional (10). Such miscoordination of pathway components (enzymes) is a major barrier for pathway engineers who hope to freely explore the biosynthetic diversity made possible by the given transformations. This is especially true for pathways constructed from highly promiscuous enzymes. In this work, when cyclases were expressed with wild-type CrtN, very low levels of cyclic C₃₅ carotenoids (compounds 11 to 14) were produced. However, tuning the step number of the desaturase by directed evolution enabled us to generate cells that produce each of these four carotenoids as the major product. Thus, altering the working environment of secondary metabolic enzymes can result in the emergence of novel pathways (8). Here, the major task of pathway engineers is to tune the coordination of the assembled components in order to unmask hidden pathways awaiting discovery. We propose that this tuning can be conducted systematically by directed evolution, a powerful optimization strategy that is applicable to many different systems, all in the absence of detailed information on individual components (36).

The C₃₅ pathway provides a unique opportunity to study the nature of carotenoid biosynthetic pathways. In this paper, we have described a route to novel C₃₅ carotenoids. This can be regarded as a consequence of uptake of a larger substrate

(GGDP) by the C₃₀ carotene synthase CrtM. Once formed, however, this “artifact” pathway recruited various downstream enzymes, and thus a full-fledged C₃₅ carotenoid pathway was established. We believe what we have observed is a model of how nature can access a variety of new metabolites in a short period of time.

An important question is whether this newly born pathway can become a mature, specific one (i.e., making only C₃₅ carotenoids). We recently demonstrated that the substrate and product specificities of a carotene synthase can be altered by a single amino acid substitution (33). However, unlike C₃₀ or C₄₀ synthase, a C₃₅-specific synthase must favor the condensation of two nonidentical substrate molecules. Can we breed a C₃₅ synthase that conducts this particular reaction, regardless of the cellular levels of FDP and GGDP? Can we force CrtM to become selective against larger prenyldiphosphates, so that it would produce only C₃₀ carotenoids, even in the presence of a high level of GGDP? Such questions can be addressed by further laboratory evolution of carotene synthases.

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