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Diversifying Carotenoid Biosynthetic Pathways by Directed Evolution

Daisuke Umeno,†‡ Alexander V. Tobias,† and Frances H. Arnold*

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

INTRODUCTION

Nature is a masterful and prolific chemist. The ever-expanding catalog of natural products from plants and microbes currently stands at more than 100,000 different compounds (58). This collection has profoundly influenced human well-being. Natural products have been used to treat pain, infections, and disease for thousands of years; their equally ancient and diverse uses as colorants, spices, fragrances, aphrodisiacs, cosmetics, and toxins have been fundamental to human culture and development. This trend shows no signs of subsiding. Of the 877 new small-molecule pharmacological entities introduced worldwide between 1981 and 2002, 61% can be traced to natural products (142). A similar proportion (59%) of the 137 small molecules in phase II or III clinical trials for cancer treatment (as of June 2003) are natural products or closely related compounds (172). Natural products and their derivatives therefore remain an essential component of the pharmaceutical industry (172). Given the wealth of functional small molecules in nature, it is perhaps not surprising that so much effort today goes into prospecting for new ones or modifying existing ones. Academic and industrial laboratories, often in massive screening programs, continue to isolate and characterize promising new compounds from biological extracts. In addition, molecular scaffolds inspired by natural products are increasingly being used as the basis for synthesizing combinatorial chemical libraries which can be screened for protein-binding or other biochemical activities (30).

Although impressive in number, the known products of natural biosynthetic pathways account for but a tiny fraction of the structures that could be produced. This essentially infinite space of possible functional molecules represents an irresistible frontier for those seeking new, bioactive compounds. For example, only 1/10 of the 877 new small-molecule pharmacological entities mentioned above are bona fide natural products—most are derivatives of natural products, synthetic compounds with natural product-derived moieties, or natural-product mimics (142). The chemical space surrounding natural products is particularly rich in biologically functional

* 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: frances@cheme.caltech.edu.

† D.U. and A.V.T. contributed equally to this work.

‡ Present address: Department of Applied Chemistry and Biotechnology, Chiba University, 1-33 Yayoi-machi, Inage-Ku, Chiba, Japan 263-8522.

molecules, and structurally related compounds can serve human purposes even better than natural products themselves. Researchers are now beginning to explore some of these nearly natural products by mixing, matching, and mutating biosynthetic genes from diverse sources in organisms such as *Escherichia coli* and screening for production of novel metabolites. In this review we illustrate how pathways can be "evolved" rapidly in the laboratory to generate new natural products. Used in this way, directed laboratory evolution is a powerful tool for discovering new pathways and for in vivo combinatorial chemistry of natural products, i.e., "combinatorial biosynthesis."

In addition to providing access to novel metabolites, laboratory evolution studies can offer insights into the natural evolution of metabolic pathways and their constituent enzymes. Comparative studies of homologous enzymes can readily identify similarities such as conserved residues and folds. However, identifying the exact genetic changes responsible for differences in the specificity, stability, or other properties of related enzymes is much more difficult. When comparing sequences that have diverged over millions of years, it is almost impossible to distinguish the handful of adaptive mutations from the plethora of differences that reflect evolutionary drift. In contrast, evolution in the laboratory allows us to capture new metabolic pathways in the act of emerging and permits the identification and analysis of the molecular events that gave rise to the new product distributions.

With carotenoid biosynthesis as our model, we describe how pathway engineers have used laboratory evolution experiments to diversify biosynthetic pathways and what they have learned from those efforts. This work has demonstrated the remarkable ability of carotenoid biosynthetic enzymes to evolve and acquire new specificities and has illuminated pathway features that facilitate the creation of new natural products based on "unnatural" molecular scaffolds. Although evolutionary pathway engineering is in its infancy, we show that it is capable of generating whole new families of natural product analogs. Some of these compounds may await discovery in nature, while others will probably never be found by natural evolution.

Overview of Carotenoid Biosynthetic Pathways

The carotenoids are a subfamily of the isoprenoids and are among the most widespread of all natural products. Carotenoids are responsible for many of the colors of animals, plants, and microorganisms and play important biological roles as accessory light-harvesting components of photosynthetic systems, photoprotecting antioxidants, and regulators of membrane fluidity. As a whole, the natural C_{30} and C_{40} carotenoid biosynthetic pathways can be organized in a tree-like hierarchy (Fig. 1), with the trunk representing the initial, universal step of backbone synthesis catalyzed by a carotenoid synthase and the outward-emanating branches and subbranches collectively denoting the various downstream modification steps seen in different species (8). The hundreds of known carotenoid biosynthetic enzymes fall into relatively few classes, based on the types of transformation they catalyze (isoprenyl diphosphate synthases, carotenoid synthases, desaturases, cyclases, etc.). The enzymes responsible for various versions of a basic transformation are related by evolution (111, 163, 176): the pathways diverged on accumulation of mutations that altered enzyme specificity. Thus, a small number of basic transformations, subtly modified in different species, collectively lead to a large number of different products, although only a handful of carotenoids (usually a few and almost always fewer than ~10) are made by any one organism.

C_{40} carotenoids are made in thousands of plant and microbial species, starting with the synthase-catalyzed condensation of two molecules of geranylgeranyl diphosphate (C_{20}PP) to form phytoene (Fig. 1). (Most phytoene synthases produce the 15*Z* isomer of phytoene [87].) Different types and levels of modification of this C_{40} backbone account for the majority of known carotenoids. C_{30} carotenoid pathways starting with the condensation of two molecules of farnesyl diphosphate (C_{15}PP) to form (15*Z*)-4,4′-diapophytoene (also called dehy-drosqualene) are much less widespread, having been found only in a small number of bacteria such as *Staphylococcus*, *Streptococcus*, *Heliobacterium*, and *Methylobacterium* species (55, 100, 101, 129, 130, 198, 205, 206). These initial C_{30} and C_{40} condensation products are dehydrogenated in a stepwise manner by desaturase enzymes, which represent the next important branch points for pathway diversification (70). In bacteria and fungi, a single desaturase catalyzes the entire sequence of carotenoid desaturation steps (32). Carotenoid desaturation in plants, algae, and cyanobacteria (not covered in this review) is accomplished by two distinct desaturases and a carotenoid isomerase (7, 31, 72, 86, 131). Desaturases from bacteria such as *Pantoea* spp. (referred to in this review by their former *Erwinia* species names, which are more commonly used than the current approved names in the carotenoid field) also catalyze isomerization of the carotenoid's central double bond, giving all-*E (trans)* desaturated products (65). With a few notable exceptions (157, 200), the desaturation level of the carotenoid backbone is well defined for each organism. In many organisms, desaturation is followed by cyclization, catalyzed by a β- or ε-cyclase and leading to carotenoids with one or two cyclized ends. A variety of further enzyme-catalyzed transformations that can include ketolation, hydroxylation, glycosylation, and oxidative cleavage act on substrates derived from the C_{30} or C_{40} backbones to produce the catalog of more than 700 known carotenoids (82).

Some organisms synthesize modified C_{40} carotenoids with additional or fewer isoprenyl units. For example, *Corynebacterium glutamicum* synthesizes "homocarotenoids" such as flavuxanthin (C_{45}) and decaprenoxanthin (C_{50}) via enzymatic prenylation of lycopene (Fig. 1) (109, 112). In contrast, several fungi produce C_{35} "apocarotenoids" such as neurosporaxanthin via oxidative cleavage of monocyclic C_{40} carotenoids (Fig. 1) (12, 14, 164, 215). Despite their non-C_{40} structures, these natural homo- and apocarotenoids are, from a biosynthetic perspective, part of the C_{40} family, since their biosynthesis proceeds via the C_{40} carotenoid backbone phytoene.

Isoprenoids and isoprenyl diphosphate building blocks are found in all organisms. *E. coli* has three isoprenyl diphosphate synthases: a farnesyl diphosphate (C_{15}PP) synthase (67, 68), an octaprenyl diphosphate (C_{40}PP) synthase (154), and a *Z*-type (see below) undecaprenyl diphosphate (C_{55}PP) synthase (94). A C_{30} carotenoid pathway can be built in *E. coli* branching directly from the organism's en-

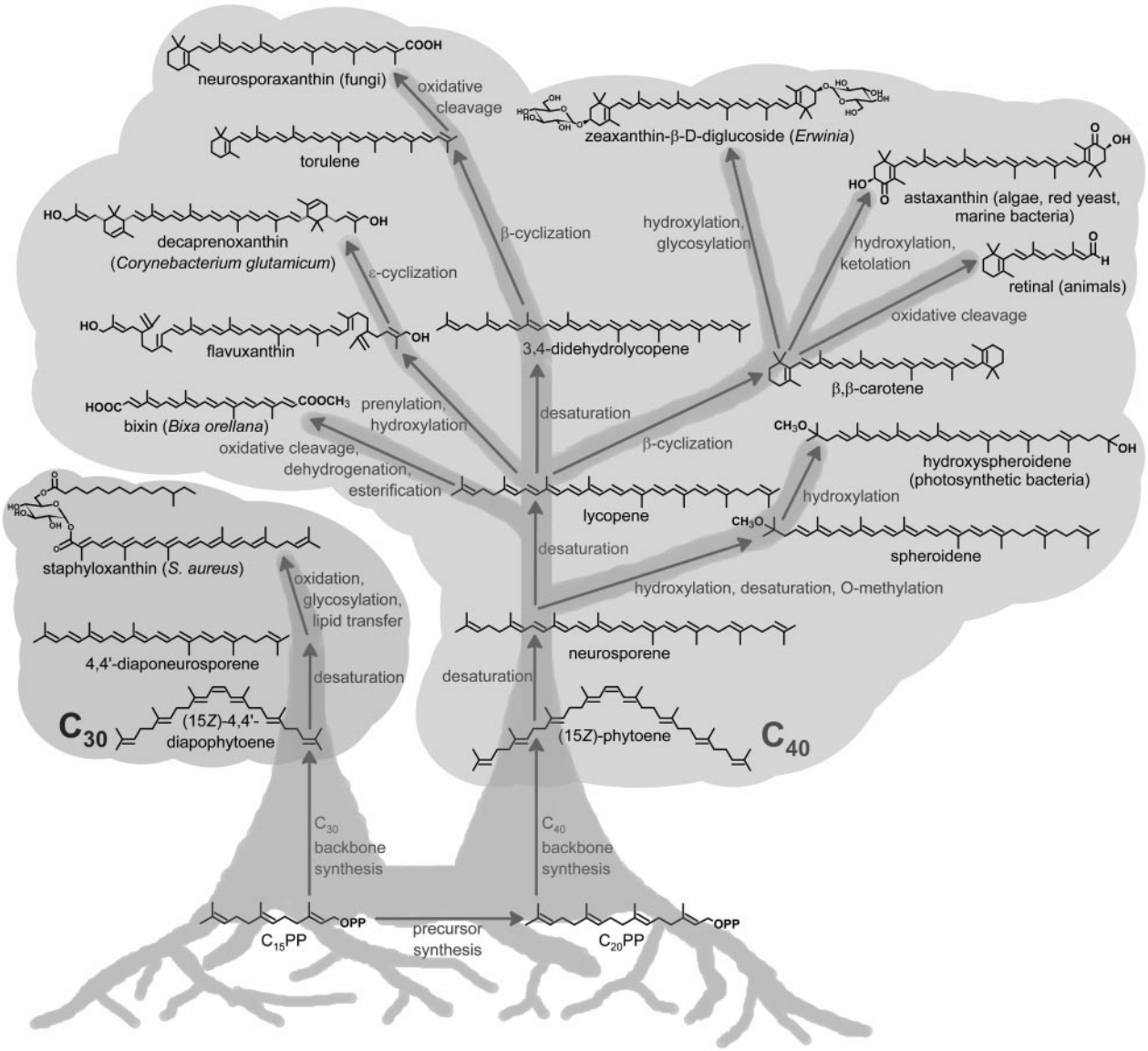


FIG. 1. Natural carotenoid biosynthetic pathways can be organized in a tree-like hierarchy. The biosynthesis of all natural carotenoids begins with the enzymatic assembly of a C_{30} or C_{40} backbone. These backbones are desaturated, cyclized, oxidized, and otherwise modified by downstream enzymes in various species-specific combinations. Shown are several common types of enzymatic transformations that occur in natural carotenoid pathways. Common carotenoids formed early in a pathway, such as lycopene, are modified in different organisms depending on the enzymes present. The multiple enzymatic routes originating from intermediates common to many end products result in extensive pathway branching (and subbranching).

dogenous C_{15}PP supply (226). However, since *E. coli* lacks a C_{20}PP synthase function, a geranylgeranyl diphosphate (C_{20}PP) synthase gene must be heterologously expressed along with the other carotenoid biosynthetic genes in order to make C_{40} carotenoids in this organism.

Most of the enzymes involved in carotenoid biosynthesis are membrane associated, and the hydrophobic intermediates and products of carotenoid pathways partition to cytoplasmic or organelle membranes, depending on the type of organism (29). It is not known whether the carotenoid biosynthetic enzymes associate into complexes. Over the years, evidence for the existence of multienzyme complexes that function as assembly lines for carotenoid synthesis has been presented (summarized in reference 32). Some of the most convincing evidence has come from experiments performed in the laboratory of Cerdá-Olmedo with the fungus *Phycomyces blakesleeanus* (6, 39, 57, 138). On the other hand, the fact that carotenoid enzymes from different organisms are readily combined to generate functional pathways in heterologous hosts is evidence that the formation of a specific enzyme complex is not a prerequisite for carotenoid biosynthesis. Throughout this review, we refer to carotenoid-synthesizing enzyme complexes when they provide a possible alternative explanation for particular observations from our laboratory and others'.

Carotenoids as a Model System for Laboratory Pathway Evolution

Carotenoid biosynthesis is particularly well suited as an experimental system for studying pathway evolution in the laboratory. Claudia Schmidt-Dannert recognized that the following features would facilitate such experiments when she first proposed to evolve these pathways as a visiting researcher in this laboratory in 1998.

Carotenoids are diverse, and their biosynthetic pathways are representative of other secondary metabolic pathways. Hundreds of carotenoids are known in nature. They are made using a few basic transformations, the earliest of which are highly conserved. The reactions involved in these early steps, such as backbone formation by carotenoid synthases and cyclization by carotenoid cyclases, are mechanistically similar to other reactions in isoprenoid biosynthesis. Modification steps later in the carotenoid pathways cover a wide range of biotransformations, many of which appear in other natural products. Thus, experience with carotenoid pathway evolution can be applied to diversifying other biosynthetic pathways.

Carotenoid biosynthetic enzymes are highly portable. The carotenoid pathway emerges directly from the central isoprenoid pathway that exists in all organisms. Thus, in principle, any organism can supply the precursors required for an engineered carotenoid pathway. Carotenoid biosynthetic genes can be expressed in a wide range of organisms to extend or redirect an existing pathway (69, 70, 105). For instance, a bacterial phytoene desaturase (CrtI) introduced into cyanobacteria (227) and tobacco plants (136) elevated their resistance to the herbicide norflurazon (known to block plant-type phytoene desaturases). Plastid-targeted introduction of an algal β,β-carotene ketolase resulted in transgenic tobacco plants that accumulated astaxanthin and changed the color of the plant's nectory tissue from yellow to red (128). The highly publicized introduction of the β,β-carotene pathway into rice resulted in substantial β,β-carotene levels in the normally carotenoid-free endosperm and therefore greater nutritional value for this food staple (234). Phytoene-producing human cells (HeLa, NIH 3T3 etc.) were created by the addition of a bacterial phytoene synthase and showed increased tolerance to oxidative shock as well as a lower rate of H-ras-induced carcinogenesis (143, 144, 179). Almost all carotenoid biosynthetic genes cloned to date, including those from plants, can be functionally expressed in *E. coli* (119, 175, 177, 180), as can animal oxidative cleavage enzymes (98, 123, 158, 220, 231). The "portability" of these enzymes greatly facilitates the assembly and evolution of novel biosynthetic pathways.

Evolution of carotenoid pathways can be tracked visually. Carotenoids are natural pigments, and their characteristic colors are ideal for product-based high-throughput screening. Scientists have made good use of this feature throughout the history of carotenoid research. Experiments involving the generation and analysis of mutant strains exhibiting altered colors have elucidated biosynthetic routes in various organisms (12, 15, 18, 40, 66, 73, 113, 120, 130, 155, 162, 173, 178, 196, 201, 216). Many carotenoid biosynthetic genes were cloned based on their ability to confer or alter color development in *E. coli* (52, 54, 133, 134, 160, 197). Carotenoid titers are an indicator of precursor levels and can be used to evaluate and tune upstream (isoprenoid) pathways (189, 223, 224). The colors generated in *E. coli* colonies provide a facile screen for new pathway products in a laboratory evolution experiment.

Carotenoids are valuable in their own right. The polyene chromophores of carotenoids, which absorb light in the 400 to 550-nm range, provide the basis for their characteristic yellow-to-red colors and their ability to quench singlet oxygen (5, 74, 79, 95, 103, 137, 218, 230). Carotenoids act as antioxidants in vivo (170, 183, 210), and many beneficial effects of carotenoids on human health have been reported, ranging from cancer prevention and tumor suppression to upregulation of immune function, reduction of the risk of coronary heart disease or age-related degeneration, and cataract prevention (19, 21, 42, 47, 83, 108). As natural pigments, carotenoids are used as colorants in foods, cosmetics, and flowers (117, 128, 209). Given these diverse and important properties of known carotenoids, we can assume that novel compounds of this family will also possess interesting biological or chemical functions.

Evolvable Pathways and Enzymes

The diversity of carotenoids and biosynthetic enzymes that exists in nature demonstrates the success of evolution in discovering and optimizing new pathways. Carotenoid pathways are evolvable and understanding the features that lead to this evolvability will allow us to design directed evolution experiments to accomplish the same goals. We define an evolvable pathway as one which can produce many new metabolites after limited genetic change. New metabolites are made following recruitment of new enzymes or functional alteration of existing ones. Such events are rare, and evolvable pathways must be particularly effective at exploiting these rare events.

In general, evolvable pathways appear to contain enzymes that are "locally specific" (32). These enzymes are not unspecific; rather, they recognize a particular structural motif common to a variety of possible substrates. Thus, if a change upstream of a locally specific enzyme generates a novel compound, there is a good chance that the enzyme will metabolize this compound further (as long as it retains the required motif) and generate a new derivative. More such enzymes further downstream will accept the new derivative(s) and produce yet more new metabolites. A carotenoid desaturase, for example, might need at most a few mutations in order to catalyze double-bond formation on a new carotenoid-like backbone. Locally specific modifying enzymes further downstream would, with high probability, accept these desaturated substrates, generating still more new compounds.

Pathway structure also contributes to evolvability: highly branched pathways can propagate discoveries along many routes. Pathway branches occur where one substrate is potentially converted to multiple products by the action of one or more enzymes. The existence of nested branches geometrically increases the number of new products that can be synthesized from a new metabolite produced upstream, provided that the downstream enzymes accept the new substrates. Evolvable and consequently diverse, carotenoid pathways are "bushy," with multiple products possible from each basic transformation. Product possibilities are dictated by the nature of the chemical reaction and substrate and ultimately by the specificity of the enzyme that controls that branch point. For example, desatu-

ration of a carotenoid backbone can produce a number of different products—the one(s) produced by any given organism reflect the specificity of its particular desaturase in its environment. That specificity can change upon mutation and can open new pathway branches.

Pathways constructed entirely of locally specific enzymes would not serve a host organism well in terms of regulation and production of tried-and-true metabolites. Thus, we expect evolvable pathways to contain more specific enzymes as well. To maximize the ease of exploring new pathways while preserving key metabolic processes, we would expect these specific enzymes to be located at the earliest steps of a pathway. Here, they can serve as molecular gatekeepers, allowing only certain primary metabolites to enter but not impeding the discovery of new structures within the pathway. Manipulating these gatekeeper enzymes to admit different substrates should be an effective way to generate whole new families of natural products.

Thus, features contributing to the evolvability of a biosynthetic pathway include the use of locally specific enzymes and biotransformations that contribute to pathway branching. More-specific gatekeeper enzymes limit what flows through the pathway, providing necessary insurance against chemical chaos. In the end, however, pathways are evolvable because their component enzymes are themselves evolvable, changing properties such as substrate and product specificity readily on mutation. Evolvable enzymes should also exhibit multiple mutational routes to a given altered phenotype and may be comparatively robust to mutation. The former property increases the chances that new phenotypes will emerge, while the latter property allows "scanning" of similar sequences without loss of function. Perhaps surprisingly, it is still an open question whether promiscuous or locally specific enzymes are more evolvable than enzymes that are highly specific. As we detail in this review, as little as a single amino acid substitution can change the specificity of highly specific carotenoid biosynthetic enzymes.

An evolvable pathway, then, would consist of an evolvable gatekeeper enzyme (or set of enzymes) coupled with locally specific downstream enzymes. Such an arrangement maximally exploits mutations in the gatekeeper enzyme, converting a single newly discovered molecule into a potentially large number of new metabolites. Several lines of evidence support the view that the enzymes involved in carotenoid biosynthesis are highly evolvable and are embedded in pathways with such evolvable structures. This review represents the first attempt at gathering together this evidence, which collectively paints a picture of carotenoid biosynthetic pathways as dynamic systems capable of exploring a diversity of product structures much greater than is seen in nature. We now examine the various ways in which this evolvability has been exploited in the laboratory to explore and extend the product diversity of these pathways.

APPROACHES TO ENGINEERING NOVEL BIOSYNTHETIC PATHWAYS

New biosynthetic pathways can be created by recruiting foreign catalytic machinery that adds a new branch; such enzyme recruitment happened frequently during the evolution of natural-product pathways (20, 27, 116). A pathway can also diversify when the function of an existing enzyme is altered by mutation or other genetic change. The wide functional diversity that exists within the evolutionarily related carotenoid pathways is evidence that enzyme evolution has played a central role in creating chemical diversity, primarily through modification of substrate and product specificity. In the laboratory, researchers can now employ the same strategies of enzyme recruitment and modification to create new biosynthetic pathways. In this review, we refer to coexpressing genes from different sources in a recombinant organism as gene assembly. This approach can be distinguished from directed evolution of the component enzymes (or their regulation), where libraries of mutant alleles are expressed along with other pathway genes in host cells, which are then screened or selected for desired properties. These two approaches generate novel compounds by mimicking the major modes of natural pathway evolution: gene transfer and divergence. Thus, the two approaches complement one another, and we consider both to fall under the umbrella of laboratory pathway evolution. In this section, we discuss efforts to create new carotenoid biosynthetic pathways by using gene assembly, directed enzyme evolution, and combined approaches.

Engineering Pathways by Gene Assembly

Assembling carotenoid biosynthetic genes from different organisms and expressing these "hybrid" pathways in a recombinant host has generated functional routes to rare or novel carotenoids (Fig. 2) (4, 5, 13, 69, 105, 118, 192, 199, 211). Where gene assembly alone successfully yielded a new pathway branch or extension, the recruited enzyme(s) usually catalyzed transformations later in the pathway. These downstream enzymes are locally specific and can accept a range of substrates. Various unusual acyclic carotenoids were obtained by assembling carotenogenic genes from *Pantoea ananatis* (*Erwinia uredovora*) and *Rhodobacter capsulatus* (4, 5). Coexpression of the C_{20}PP synthase CrtE and the phytoene synthase CrtB from *E. uredovora* along with various combinations of three different carotenoid desaturases, a carotenoid hydratase, a β-cyclase, and a hydroxylase resulted in the production of four previously unidentified carotenoids, including 1-OH-3′,4′-didehydrolycopene with potent antioxidant activity (5). In another example, addition of CrtW, a β-end ketolase from *Paracoccus* sp. strain MBIC1143 ("*Agrobacterium aurantiacum*"), extended the zeaxanthin β-D-diglucoside pathway from *Erwinia*, leading to synthesis of novel compounds astaxanthin β-D-diglucoside and adonixanthin 3′-β-D-glucoside (Figure 2) (235). As we show in later sections, other downstream carotenoid-modifying enzymes are also locally specific and should be similarly useful for "combinatorial biosynthesis" of novel carotenoids by gene assembly. We predict that systematic combinatorial assembly of these genes will result in the biosynthesis of many hundreds of new carotenoids.

Assembling locally specific enzymes in a recombinant organism can sometimes result in an interconnected "matrix" pathway that leads to numerous carotenoid structures, as illustrated in Fig. 3 (135). However, many of the intermediates or end products may not be accessible due to imbalances in enzyme expression, activity, or specificity. Thus, a few dominant compounds may be produced, while other (possible) ones remain hidden (but poised to appear under different conditions). Furthermore, it can be difficult to predict the relative abundances

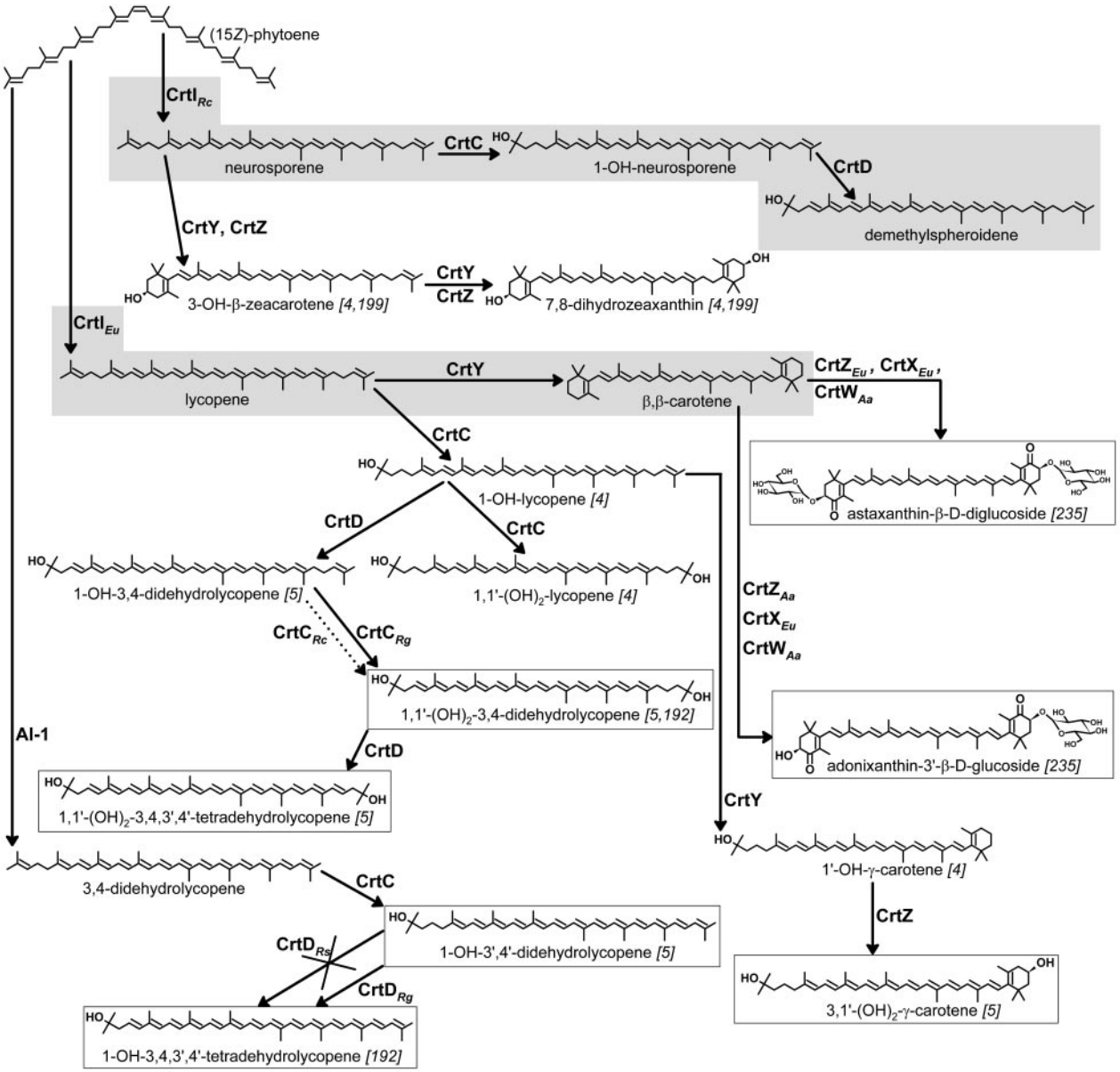


FIG. 2. Selected examples of gene assembly leading to novel carotenoids in *E. coli.* Combining enzymes from different source organisms can lead to rare or novel carotenoids. In some cases, such as with CrtC and CrtD (4, 5), new pathway branches can be uncovered by replacing an enzyme with its counterpart from a different organism (192). Carotenoids in boxes had not been previously identified in biological material or chemically synthesized. Enzyme abbreviations: AI-1, phytoene desaturase from *Neurospora crassa* (five-step); CrtC, neurosporene 1,2-hydratase; CrtD, 1-OH-neurosporene 3,4-desaturase; CrtI$_{Eu}$, phytoene desaturase from *Erwinia uredovora* (four-step); CrtI$_{Rc}$, phytoene desaturase from *Rhodobacter capsulatus* (three-step); CrtW, β,β-carotene 4,(4′)-ketolase; CrtX, zeaxanthin glycosylase; CrtY, lycopene β-cyclase; CrtZ, β,β-carotene 3,(3′)-hydroxylase. Other enzyme subscripts: *Aa*, "*Agrobacterium aurantiacum*" (current designation, *Paracoccus* sp. strain MBIC1143); *Eu*, *Erwinia uredovora* (current approved name, *Pantoea ananatis*); *Rg*, *Rubrivivax gelatinosus*; *Rs*, *Rhodobacter sphaeroides*. Natural pathways leading to the synthesis of demethylspheroidene and β,β-carotene are shaded. The dotted arrow represents trace enzymatic conversion, and the crossed-out arrow denotes undetectable enzymatic conversion. References to original publications describing the biosynthesis of various compounds are given in brackets beside compound names.

of the different metabolites. For example, various attempts to construct a pathway for astaxanthin synthesis in a heterologous host resulted in quite different proportions of astaxanthin and intermediates (76, 135). In another example, introduction of the carotenoid biosynthetic enzymes CrtB, CrtI, CrtY, and CrtZ from *Pantoea agglomerans* (*Erwinia herbicola*) into a desaturase-deleted mutant of *Rhodobacter sphaeroides* merely restored desaturation activity and failed to alter the range of carotenoids produced (85). This occurred despite the introduction of a β-cyclase (CrtY) and a β-end hydroxylase (CrtZ) into a host lacking these catalytic functions. Only when the endogenous neurosporene hydroxylase gene (*crtC*) was deleted was a functional pathway to cyclized and hydroxylated carotenoids realized (69, 70). In this case, the endogenous CrtC in *R. sphaer-*

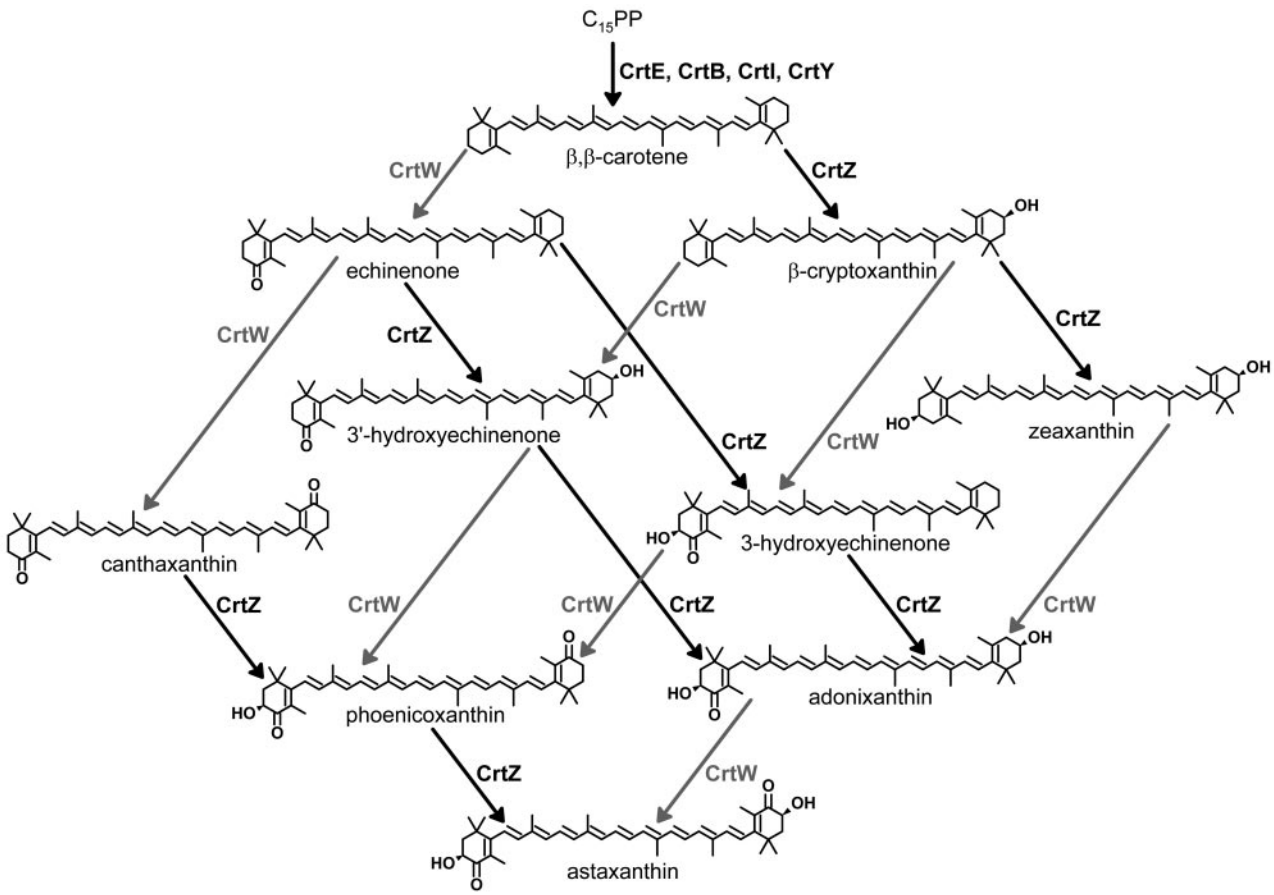


FIG. 3. "Matrix" pathway resulting from the coexpression of carotenoid biosynthetic enzymes with broad specificity. Coexpression in *E. coli* of CrtW [β,β-carotene 4,(4′)-ketolase] and CrtZ [β,β-carotene 3,(3′)-hydroxylase] from "*Agrobacterium aurantiacum*" (current designation, *Paracoccus* sp. strain MBIC1143) along with the genes for β,β-carotene synthesis from *E. uredovora* (current approved name, *Pantoea ananatis*) resulted in the biosynthesis of at least nine different β,β-carotene derivatives (135). This figure was made using data from reference 135.

oides may have been so active that the desaturase CrtI, cyclase CrtY, and β-end hydroxylase CrtZ introduced from *E. herbicola* could not compete with it for neurosporene. Another possible explanation is that incorporation of CrtC into a carotenoid biosynthetic enzyme complex blocked the additional inclusion of CrtY, CrtZ, and more than three desaturase subunits. These examples illustrate the importance of properly coordinating pathway enzymes in order to create a specific pathway to a desired product. This problem of miscoordination makes it exceedingly difficult to systematically explore all the possible metabolites of a given set of enzymes solely by gene assembly. A major task of pathway engineers will be to coordinate assembled components in order to unmask hidden metabolites. This can be accomplished by directed evolution.

Engineering Pathways by Directed Enzyme Evolution

Nature has generated a vast number of biosynthetic genes by mutation, recombination, and natural selection. Variations in properties such as desaturation step number or synthase specificity contribute enormously to the diversity of carotenoid structures seen in nature. In the previous section, we presented examples of how gene assembly alone can lead to novel or unusual carotenoids. However, we can also consider creating enzyme variants with desired specificities (or other properties such as expression level or stability) by evolving them in the laboratory. This approach circumvents the need to find a specific function in nature and reproduce that function in a heterologous host. Instead, enzymes with the desired properties are found by screening libraries of mutants of an enzyme that performs the same type of chemical transformation.

Some enzyme specificities not available in nature become readily available by evolution in the laboratory, opening pathways to new metabolites. Decoupled from the biological constraints imposed by the need to fulfill a particular function throughout the course of evolution, a pathway can in principle explore all chemically accessible products, not just those that are biologically relevant (10). Thus, directed evolution gives us the ability to explore the space of possible chemical products much more rapidly and also more thoroughly than does natural evolution. By evolving biosynthetic enzymes, we can anticipate the discovery of large numbers of new natural products on the benchtop in convenient laboratory hosts.

Although each directed evolution project is unique, all involve two main steps: making a library of mutants and searching that library for desired properties. The mutant library is typically generated in an error-prone PCR amplification (38, 124) tuned to generate a certain average point mutation rate or

by DNA shuffling, a method that makes new point mutations and recombines existing mutations (193) (many methods are available for making libraries of mutant genes for directed evolution; useful protocols are given in reference 9). The library of mutated DNA molecules is then subcloned into an appropriate vector for expression with other pathway genes in a convenient host organism such as *E. coli*. Hundreds or thousands of clones are screened in search of (typically) rare ones expressing favorably altered enzymes. A powerful aspect of this process is that it can be iterated—improved variants can be subjected to further rounds of mutagenesis and screening, often leading to further improvements. A sensitive, reliable screen is key to a successful directed evolution experiment: the screen allows the researcher to identify the typically rare mutants with new and interesting properties.

Different strategies exist for evolving metabolic pathways in the laboratory. In an early study, multiple genes in an arsenate degradation pathway were subjected to mutagenesis and recombination all at once (49). This "fully blind" approach makes the most sense when little is known about which genes or noncoding regions (such as ribosomal binding sites) should be mutated to obtain a desired phenotype. However, a significant disadvantage of this approach is that the combinatorial complexity (the size of the library) increases concomitantly with the length of DNA targeted for mutation. Consequently, more clones must be screened to find the rare ones with improved properties. Therefore, when possible, it is attractive to target individual genes for mutation and then screen those mutants in the context of the whole pathway; this can be thought of as a "partially blind" approach. Carotenoid biosynthetic pathways are sufficiently well characterized that investigators generally know which enzyme to target for mutagenesis in order to achieve a new product distribution; however, the specific mutation(s) needed is unknown. Accordingly, researchers have thus far chosen to extend carotenoid pathway branches in a stepwise manner by evolving one gene at a time and expressing the library of variant alleles together with other wild-type or previously laboratory-evolved pathway genes. Schmidt-Dannert et al. demonstrated the effectiveness of this approach for diversifying the range of carotenoid structures accessible from a given set of enzymes (181).

PROBING THE EVOLVABILITY OF CAROTENOID BIOSYNTHETIC ENZYMES AND PATHWAYS

In the Introduction, we reasoned that highly evolvable pathways would be assembled from locally specific downstream enzymes and (a small number of) more specific "gatekeeper" enzymes. Furthermore, the enzymes themselves should readily alter specificity on mutation. Modifying a gatekeeper enzyme, accompanied by fine-tuning of downstream enzymes where needed, could allow whole new families of natural products to emerge. In this section, we assess the evolvability of carotenoid biosynthetic pathways and enzymes in view of the (laboratory) data now available.

Directed Evolution of Key Carotenoid Biosynthetic Enzymes

Isoprenyl diphosphate synthases. Isoprenyl diphosphate synthases (IDSs) catalyze the consecutive condensation of five-carbon isopentenyl diphosphate with allylic diphosphates to generate the precursors to all isoprenoid compounds. A variety of IDSs supply the building blocks for the >23,000 known isoprenoids ranging in size from C_5 to $C_{\sim 2,500}$ (48, 146). IDSs catalyze the 1′–4 condensation of an allylic diphosphate (C_{5n}PP) with isopentenyl diphosphate, producing the next incremental isoprenyl diphosphate ($C_{5(n+1)}$PP) (Fig. 4). This process continues until the growing isoprenoid chain reaches a certain length, at which point the reaction terminates and the product is released from the enzyme. There exist *E*-type IDSs that synthesize products with precise all-*E (trans)* double-bond stereochemistry as well as Z-type IDSs that catalyze the formation of *cis*-double bonds in the growing isoprenyl chain. IDS enzymes are quite specific in their product size and are often classified on this basis. Elucidating the molecular mechanisms of chain length control has been a major scientific interest in the study of this class of enzymes; for reviews, see references 97, 122, 145, and 225.

Although IDSs make specific products, minor modifications can change this specificity. Ohnuma et al. randomly mutated the farnesyl diphosphate (C_{15}PP) synthase gene from *Bacillus stearothermophilus* (*bstFPS*), coexpressed this mutant library along with phytoene synthase (*crtB*) and phytoene desaturase (*crtI*) genes in *E. coli*, and looked for colonies that produced the red C_{40} carotenoid, lycopene (152). Most of the colonies were colorless because they lacked the C_{20}PP precursor needed by CrtB to make carotenoids. However, a small fraction of the colonies were pink due to lycopene production; these harbored mutant C_{15}PP synthases capable of synthesizing C_{20}PP. In further work, the same group generated additional variants of BstFPS that made even longer products (150). Some of the double and triple mutants of this enzyme yielded products as large as $C_{>100}$PP. In another study, Ohnuma et al. converted an archaebacterial C_{20}PP synthase into C_{25}PP and C_{30}PP synthases by mutagenesis coupled with complementation screening in a yeast strain deficient in C_{30}PP synthesis (148). In all the above cases, analysis of mutants making larger products revealed substitutions for smaller amino acids at a position 5 residues upstream of the first aspartate-rich motif (FARM) of the enzyme (151). Aided by the crystal structure of *Gallus gallus* (chicken) C_{15}PP synthase (204), Tarshis et al. independently found a similar result with this enzyme (203). Site-directed mutagenesis at the fifth (F112) and fourth (F113) residues upstream of the FARM showed that smaller amino acids at these positions yielded enzymes that produce longer products. Subsequent work also demonstrated the converse: replacement of chain-length determining residues upstream of but close to the FARM with larger amino acids leads to shorter products. For example, *Sulfolobus acidocaldarius* C_{20}PP synthase was rationally converted into a C_{15}PP synthase by using this approach (149). The C_{15}PP synthases from *B. stearothermophilus* and *G. gallus* were converted to geranyl diphosphate (C_{10}PP) synthases by the same strategy (61, 141).

This strategy of substituting residues just upstream of the FARM with smaller or larger amino acids has also been applied to a plant short-chain (C_{20}PP) IDS (104), heterodimeric medium-chain (C_{30}PP and C_{35}PP) IDSs (78, 81, 238), as well as long-chain (C_{40}PP-C_{50}PP) synthases (75, 153) to alter the product chain length. In several cases, substitution of residues lining the substrate binding pocket but distant from the FARM (or

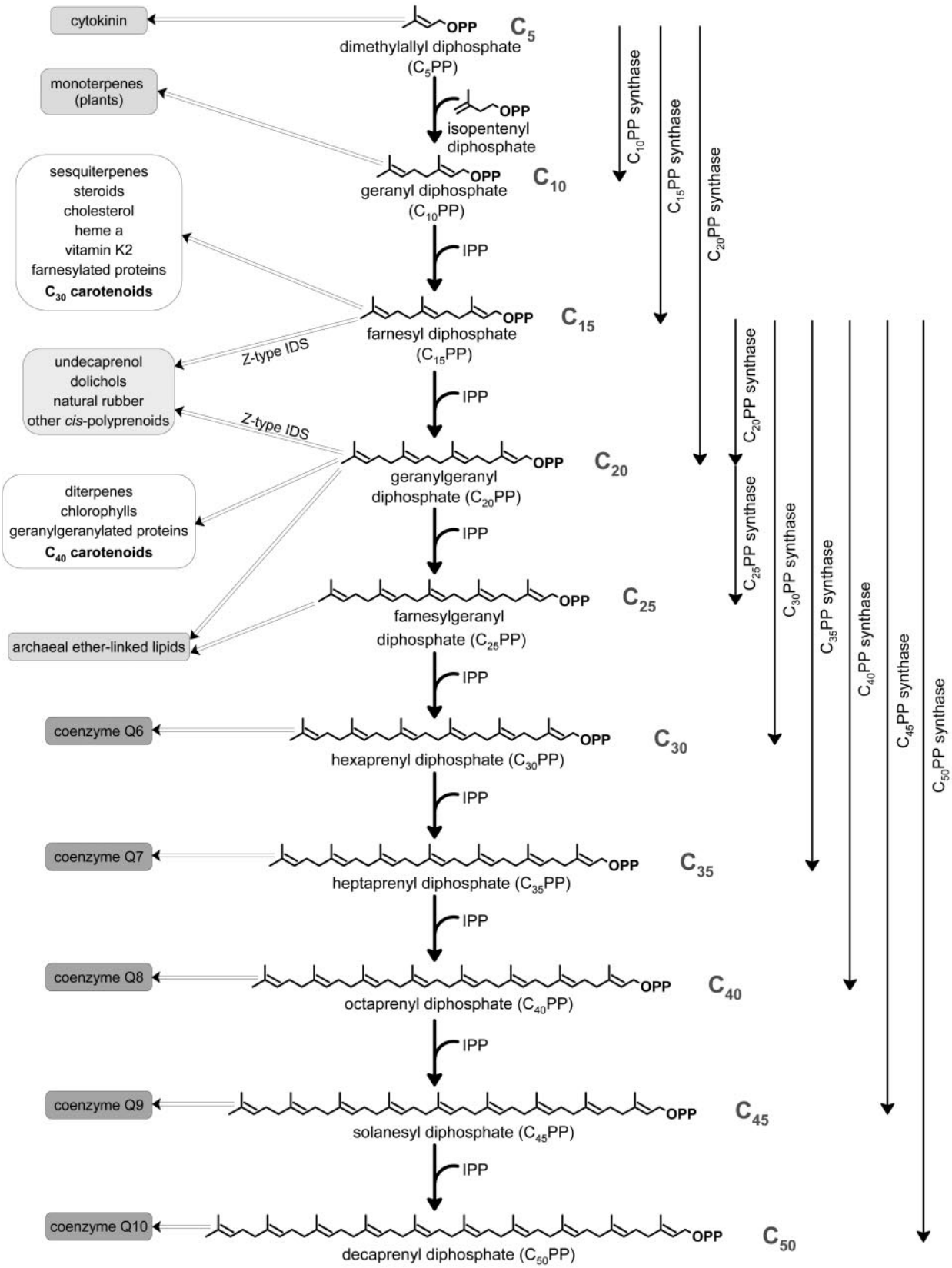


FIG. 4. Chain elongation reactions catalyzed by natural all-*E* IDSs. Each IDS adds a defined number of IPP molecules to an allylic diphosphate substrate before the final product is released. Wild-type IDSs have quite stringent product specificity; catalytic ranges of known all-*E* IDSs are shown with arrows on the right. Natural products derived from the various isoprenyl diphosphates are shown on the left. This figure was made using data from reference 225.

even located on the opposite subunit in heterodimeric enzymes) has also modified the product chain length (75, 77, 80, 92, 96, 148, 152, 238, 239). Many of these substitutions enhance the effects of mutations upstream of the FARM, leading to even greater changes in the product chain length.

Several C_{15}PP synthases have some degree of substrate tolerance (56, 107) and can be used for stereospecific synthesis of various insect hormones and related analogs (102, 106, 126). BstFPS was found to accept a number of nonnatural substrates (140), and its substrate range was further broadened by substitutions at Y81, previously described as a chain length-determining residue (151) (see above). The Y81A variant of BstFPS, for example, accepts various isoprenyl diphosphate analogs with ω-oxygen atoms in their prenyl chains as substrates, leading to the enzymatic synthesis of butterfly hair pencil pheromone and analogs (125). In theory, all of these isoprenoid diphosphate analogs could serve as building blocks for the enzymatic synthesis of carotenoid analogs.

IDSs from diverse species have exhibited evolvability in the laboratory. Although the wild-type IDSs are quite specific with respect to product size, minor genetic changes were found to profoundly alter the number of elongation steps and therefore the size of the products formed by these enzymes. The residues that were found to change the specificity of these IDSs upon mutation form a key part of the reaction pocket that accommodates the elongating isoprenoid chain, and it appears that product specificity is largely dependent on the size of this pocket (61, 203). Inspection of the data from the reports describing the modification of product specificity reveals that, in general, the mutant IDSs have a broadened rather than a shifted product range. In most cases, mutants synthesize multiple new products as well as the old one. In contrast, wild-type IDSs, although related by evolution, are very specific with respect to the length of their products. We now know that a single amino acid substitution can broaden the product range of an IDS. However, we do not know the degree of genetic change required to completely shift IDS product specificity such that it synthesizes only isoprenyl diphosphates of a new length.

Carotenoid synthases. Carotenoid synthases catalyze the synthesis of a carotenoid backbone from isoprenyl diphosphate precursors. Various phytoene (C_{40}) synthases have been isolated from organisms ranging from bacteria to higher plants. Only one C_{30} carotenoid synthase has been cloned and sequenced to date: *crtM* from *Staphylococcus aureus* (226).

It has long been recognized that the enzyme-catalyzed reaction leading to formation of the carotenoid backbone is very similar to the reaction catalyzed by squalene synthase (SqS), the first committed enzyme in cholesterol biosynthesis (32). Carotenoid synthases and SqS employ the same mechanism, and SqS produces the C_{30} carotenoid backbone if deprived of NADPH (88, 202, 237). Carotenoid synthases and SqS also share several highly conserved domains, and it is likely that the two have a common evolutionary origin (176).

The biosynthesis of carotenoid backbones (and squalene) has proven to be a complex process (22, 88, 89) (Fig. 5a). The reaction proceeds in two distinct steps. The first consists of abstraction of a diphosphate group from a prenyl donor followed by 1-1′ condensation of the donor and acceptor and loss of a proton to form a stable cyclopropyl intermediate. In the second step, the cyclopropyl intermediate is rearranged, the second diphosphate group is lost, and the resultant carbocation is quenched after the loss of another proton (Fig. 5a). Biochemical (156) and structural (159) studies have shown that

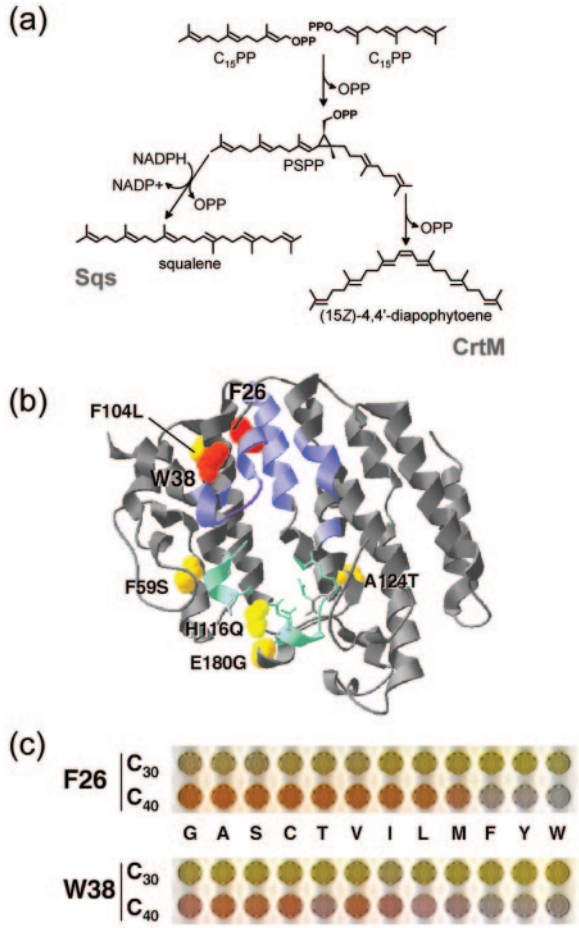


FIG. 5. Product length determination by CrtM and mutants thereof. (a) Reaction mechanism of squalene synthase (left pathway) and CrtM (right pathway). Both enzymes catalyze the head-to-head condensation of two molecules of C_{15}PP to yield linear C_{30} hydrocarbons. The enzymes employ similar mechanisms involving a common stable intermediate, presqualene diphosphate (PSPP). The mechanisms differ only in the final rearrangement step, which is followed in the case of squalene synthase by reduction of the central double bond. (b) Crystal structure of human squalene synthase (PDB ID 1EZF). Green residues are involved in the first half-reaction (formation of PSPP), while blue residues form the pocket of the second half-reaction in which PSPP is rearranged. Red residues correspond by sequence alignment to F26 and W38 of CrtM, which were found to control the product size of that enzyme (213, 214). Shown in yellow are the residues that align with mutations in CrtM that improve both its C_{30} and C_{40} carotenoid synthase activities. (c) Mutation analysis of F26 and W38 of CrtM. Various site-directed variants of CrtM (indicated by their one-letter amino acid code) were constructed and tested for their synthase activity in both C_{30} and C_{40} carotenoid pathways. Variants are arranged from left to right in increasing order with respect to the van der Waals' volume of the substituted amino acid. The C_{30} carotenoid synthase activity of each variant was estimated from the level of yellow pigmentation when coexpressed with CrtN. Similarly, the C_{40} carotenoid synthase function of each variant was evaluated in terms of the level of red pigmentation when coexpressed with CrtE and CrtI. Panels a and b were made using data from reference 212.

these two subreactions occur in physically distinct sites in the enzyme (Fig. 5b).

Carotenoid synthases are quite specific with respect to product size, which reflects a strong preference for prenyl diphosphate substrates of a particular length. The C_{40} carotenoid synthase CrtB does not detectably accept C_{15}PP as a substrate, while the C_{30} synthase CrtM accepts two molecules of C_{20}PP to form C_{40} carotenoids much less efficiently than it accepts two molecules of C_{15}PP to form C_{30} carotenoids (163, 214). Even though various longer and shorter isoprenyl diphosphates are made by different organisms (146), no known carotenoids are derived from them. Terpene synthases also utilize specific precursors (41), although product specificity can be quite modest (190).

Carotenoid synthases, positioned early in the biosynthetic pathway and possessing the ability to discriminate between isoprenyl diphosphate substrates with different numbers of isoprene units, appear to play the role of pathway gatekeeper, particularly in organisms in which multiple potential substrates are available. Indeed, as we illustrate, downstream carotenoid biosynthetic enzymes are less specific than the synthases and appear to recognize only a particular motif of the substrate. In the Introduction, we reasoned that gatekeeper enzymes should be attractive targets for pathway diversification. If carotenoid synthases could be engineered to accept different prenyl substrates and synthesize carotenoid backbones from them, the locally specific downstream enzymes might accept these new backbones and generate the corresponding metabolites. This would give rise to whole new pathway families. Compared to the IDSs, however, little is known about the molecular basis for the substrate and product specificity of carotenoid synthases.

Following the lead of those who studied the IDSs (96, 122, 125, 141, 147, 148, 150–152, 203, 238, 239), we probed the evolvability of size specificity in the carotenoid synthases by looking for mutants that complement a non-native pathway. Using error-prone PCR to perform random mutagenesis, expressing the mutant genes with the remaining genes necessary to produce lycopene in *E. coli*, and screening to find red colonies, we identified single amino acid substitutions in the *S. aureus* C_{30} carotenoid synthase CrtM (F26L or F26S) that confer the ability to make the C_{40} backbone just as efficiently as CrtB (214). Repeating this experiment using a PCR method designed to guarantee that the libraries would be free from mutation at F26 allowed us to uncover two additional amino acid substitutions, W38C and E180G, that also confer C_{40} function on CrtM (213). Mapped onto the crystal structure of human squalene synthase (SqS [Fig. 5b]), F26 and W38 appear in helices B and C, respectively, and the side chains of both residues point into the pocket that accommodates the second half-reaction (rearrangement of the cyclopropyl intermediate). Replacement of these amino acids with smaller ones significantly increased the C_{40} synthase activity of CrtM (Fig. 5c). Conversely, the C_{30} synthase performance was the highest for wild-type CrtM and decreased with decreasing size of the amino acid residues at these positions. This analysis led to our proposal that wild-type CrtM is able to perform the rate-limiting first half-reaction of phytoene synthesis—condensation of two molecules of C_{20}PP to form the cyclopropyl intermediate, prephytoene diphosphate—but the second half-reaction is prevented from going to completion by steric inhibition of the normally fast rearrangement step. Unable to convert into phytoene in wild-type CrtM, prephytoene diphosphate either remains stuck in the enzyme, departs, or undergoes other types of rearrangement to yield noncarotenoid products. (A similar phenomenon is well known for SqS; in the absence of NADPH, which is required to convert presqualene diphosphate to squalene, SqS produces a complex mixture of nonsqualene compounds including rillingol, 10-hydroxybotryococcene, and 12-hydroxysqualene [22, 89].) However, in CrtM mutants where F26 or W38 is replaced with a smaller or more flexible amino acid, the prephytoene diphosphate formed from two molecules of C_{20}PP is efficiently rearranged to form phytoene. Thus, the F26 and W38 mutations apparently modify the product specificity, not the substrate specificity of CrtM: they do not act by allowing the enzyme to bind and accept GGPP as a substrate but, rather, allow the intermediate prephytoene diphosphate to be converted to phytoene.

Gain of C_{40} function by mutagenesis of CrtM usually came at a cost to the original C_{30} synthase activity. For example, some enzymes doubly mutated at F26 and W38 showed only negligible C_{30} synthase activity. However, other modes of obtaining C_{40} synthase activity were possible. The E180G substitution increased performance in both the C_{30} and C_{40} contexts (212). Mapped onto the SqS structure, E180G is positioned outside the reaction pocket but close to the site of the first half-reaction. We think this mutation accelerates the rate-limiting first half-reaction. Several such activating mutations were found by random mutagenesis of $CrtM_{F26S}$ in a successful search for improved or restored C_{30} function (D. Umeno, unpublished results). All of these activating mutations were rather far from the reaction center (Fig. 5b, indicated in yellow) and enhanced both C_{30} and C_{40} synthase activity. These mutations could be increasing expression level, stability (half-life in vivo), or specific activity.

Like IDSs, carotenoid synthases appear to be specific, but at least in the case of CrtM, that specificity is readily altered by mutation. Furthermore, there are multiple mutational routes to the same altered phenotype. In contrast, in similar experiments with CrtB, we were unable to find any mutations that conferred C_{30} carotenoid synthase function. We are tempted to speculate that CrtM, by virtue of the fact that it does not need to select C_{15}PP from C_{20}PP in its natural host (C_{20}PP is not made in C_{30} carotenoid-producing organisms), is inherently more evolvable toward accepting C_{20}PP than is CrtB toward accepting C_{15}PP, because CrtB has always had to select C_{20}PP over C_{15}PP (the latter is present in all organisms). CrtM can in fact accept C_{20}PP in a hybrid reaction: when supplied with both C_{15}PP and C_{20}PP, wild-type CrtM can condense one molecule of each to synthesize a C_{35} carotenoid backbone (see below). CrtM has thus proven to be an evolvable enzyme while CrtB has not, at least with respect to the specific tasks of accepting a larger (CrtM) or smaller (CrtB) substrate. As was the case with the IDSs, a single amino acid substitution was sufficient to increase the size of the products synthesized by CrtM. Furthermore, as we describe below, the mutants of CrtM that function in a C_{40} carotenoid biosynthetic pathway are also capable of synthesizing even larger carotenoid backbones when supplied with the appropriate precursors.

Carotenoid desaturases. Phytoene and 4,4′-diapophytoene, the (colorless) products of the natural carotenoid synthases,

have three conjugated double bonds in the center of their backbones. Formation of carotenoid pigments requires extension of this conjugated double-bond system. The photochemical properties of a carotenoid (including its color) depend strongly on the size of the chromophore and therefore on the number of desaturation steps catalyzed by the desaturase enzyme(s). A C_{40} backbone can accommodate up to 15 conjugated double bonds, corresponding to six sequential desaturation steps. C_{30} carotenoids can undergo at most four desaturation steps (11 conjugated double bonds).

In general, bacterial C_{40} desaturases are functional on C_{30} carotenoids and vice versa. It is proposed that carotenoid desaturases recognize only a portion of the substrate molecule common to both C_{30} and C_{40} carotenoid backbones (163, 174). Subsequent carotenoid-modifying enzymes are also often locally specific (see below). Because each of the desaturation intermediates represents a branch point for further diversification by downstream enzymes, altering and controlling the desaturation step number is key for creating extensive molecular diversity (70). Nature has done this: many different carotenoid desaturases are known in C_{40} pathways, each with its specific step number. Carotenoid desaturases which primarily catalyze two, three, four, and five desaturation steps are known; one-step and six-step desaturated carotenoids have been reported only as minor products in natural carotenogenic organisms. The ability of carotenoid desaturases to accept different substrates was demonstrated when C_{30} and C_{40} desaturases were tested in each other's pathway (163, 214). C_{40} desaturases from *Erwinia* (four-step enzymes), *Rhodobacter* (three-step), and *Anabaena* (two-step) are all active on C_{30} substrates. Similarly, the C_{30} desaturase CrtN showed measurable activity in a C_{40} pathway. The localized specificity of these desaturases has been exploited in engineered pathways (211) (see below).

The desaturase step number can be inferred from the color (or color change) of the carotenoids produced in vivo. This provides an excellent basis for screening mutant desaturases in the laboratory for the ability to accept new substrates or for changes in product specificity. Using this principle, Schmidt-Dannert et al., working in this laboratory, readily isolated desaturase variants with altered step number in a C_{40} pathway (181). Starting from a library made by DNA shuffling of two closely related four-step phytoene desaturases (*crtI* from *P. agglomerans* [*E. herbicola*] and *crtI* from *Pantoea ananatis* [*E. uredovora*]), they isolated one four- to six-step variant, $CrtI_{14}$ (Fig. 6), as well as 20 variants that catalyze fewer (less than four) steps. Wang and Liao conducted similar experiments with the three-step desaturase from *Rhodobacter sphaeroides* (222). Two rounds of random mutagenesis by error-prone PCR and color screening resulted in variants that accumulated the four-step product, lycopene. At least five different mutations increased the step number of this desaturase. Furthermore, some combinations of mutations resulted in yet higher production of lycopene (222).

We recently investigated the ability of the C_{30} desaturase CrtN from *S. aureus* to alter its product specificity on mutation. CrtN is a three-step desaturase in *S. aureus* (226). *E. coli* colonies expressing this enzyme together with the C_{30} synthase CrtM develop a yellow-orange color due to the production of 4,4′-diaponeurosporene (three steps) and 4,4′-diapolycopene (four steps). When *E. coli* were transformed with a library of *crtN* mutants made by error-prone PCR, the variation in the colors of the colonies (Fig. 7), from pale to weakly fluorescent, lemon, yellow, orange, and red, was striking. To our surprise, approximately 30% of the colonies were lemon or yellow, different from the wild type. These were shown to produce 4,4′-diapophytofluene (one-step desaturation) and 4,4′-diapo-ζ-carotene (two steps) (Umeno, unpublished). We also isolated a large number of red mutants, which were found by high-performance liquid chromatography analysis to produce mainly 4,4′-diapolycopene (four steps) (Fig. 7).

Multiple experiments have thus shown that bacterial carotenoid desaturases are evolvable. The high frequency of color variants in the mutant libraries suggests that there are multiple mechanisms by which the step number can change upon mutation. It is possible that most (if not all) mutants with altered desaturation step number have acquired this change in product specificity by up- or down-regulating total desaturase activity rather than by altering intrinsic enzyme specificity, which we would expect to occur only rarely. The higher frequency of mutants with lower desaturation number compared to mutants with elevated desaturation number supports this idea, because there are many more paths downward with respect to a property such as activity or expression level. Many mutant desaturases with elevated step numbers turned out to have mutations near a putative flavin adenine dinucleotide (FAD)-binding domain (181, 222), indicating that FAD access might be a source of step number control.

Carotenoid desaturases tend to exhibit more well-defined product specificity in their natural hosts than when (over)expressed in a heterologous host. For instance, CrtN appears to be a three-step enzyme and produces almost exclusively 4,4′-diaponeurosporene in *S. aureus* (226). In a heterologous host, however, its desaturation step number is less distinct. Several groups have reported that CrtN produces the four-step 4,4′-diapolycopene as a major product in an *E. coli* system (118, 163, 211, 214). Desaturase step number can also be altered by manipulation of enzymes further downstream in the carotenoid pathway (69). It is not surprising that altering the environment of a desaturase can alter its product specificity. If downstream enzymes that normally remove certain desaturation products are not present, the desaturase may have an opportunity to catalyze further desaturation steps. In addition, if these enzymes associate in complexes, expression of a desaturase with other carotenoid biosynthetic genes from different organisms could yield complexes with altered or suboptimal substrate transfer properties, which could affect the desaturation step number. Further research should shed light on the source of desaturase specificity and on the ways in which specificity can be altered.

Carotenoid cyclases. Cyclization is another important branch point for carotenoid diversification. Cyclic carotenoids are produced in plants, algae, and photosynthetic bacteria, and enzymes catalyzing the formation of different cyclic products have been characterized (11, 52, 54, 84, 109–111, 194). A given cyclase usually produces only one kind of ring structure (32). However, an exception was recently reported by Stickforth et al., who showed that the enzyme CrtL-*e* from the cyanobacterium *Prochlorococcus marinus* is both a β- and an ε-cyclase (194). The cyclases share nearly identical mechanisms, differing only in the final rearrangement step (Fig. 8a).

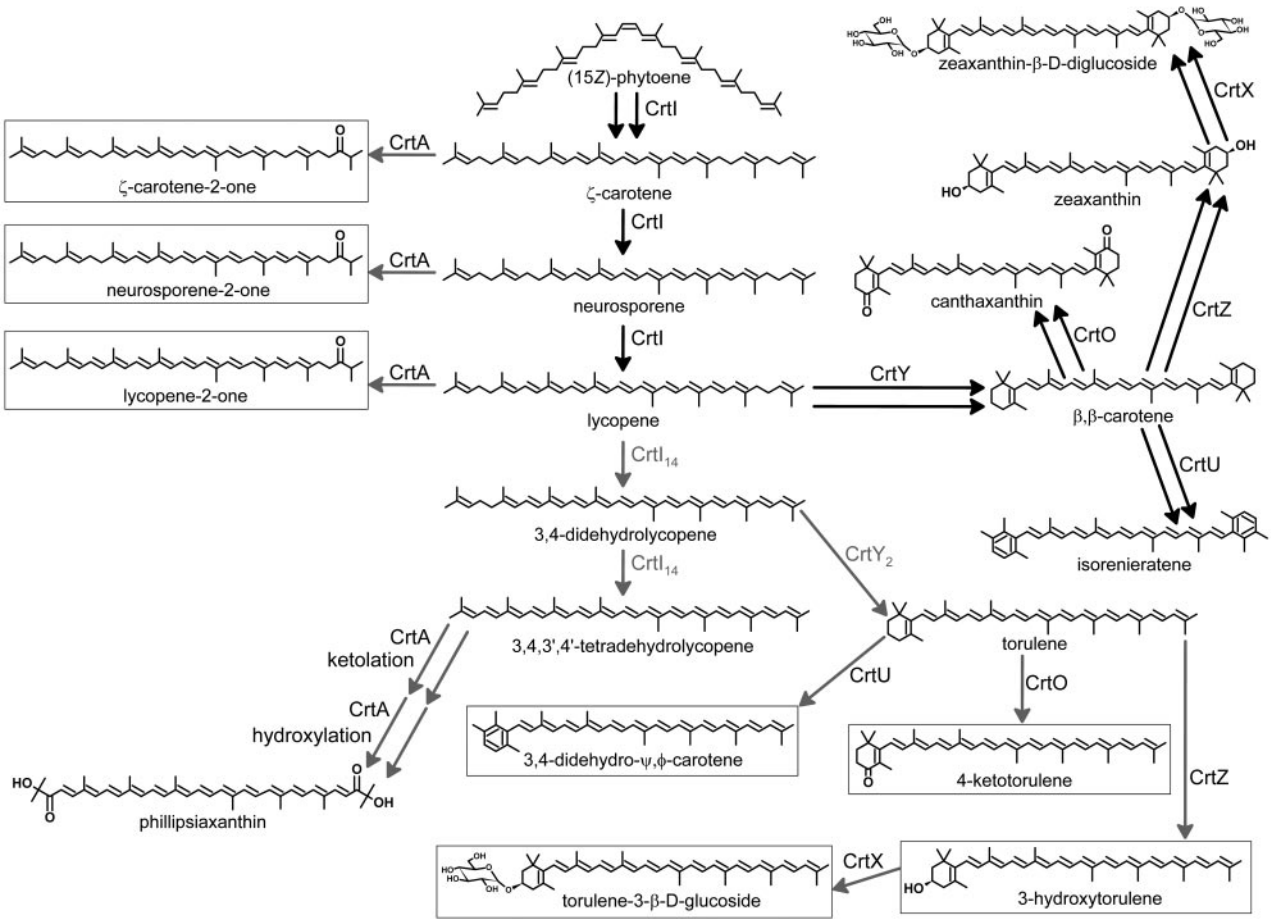


FIG. 6. Extension of laboratory-evolved pathways to 3,4,3′,4′-tetradehydrolycopene and torulene by coexpression of downstream carotenoid modifying enzymes. Boxed structures had not been previously identified in biological material. Native enzyme reactions are shown with black arrows. Gray arrows depict reactions not seen in natural organisms, and laboratory-evolved enzymes are written in gray lettering. Double arrows indicate enzymatic modification of both ends of a carotenoid substrate. Enzyme abbreviations: CrtA, spheroidene monooxygenase; CrtI, phytoene desaturase from *E. uredovora* (current approved name, *Pantoea ananatis*) (four-step), $CrtI_{14}$, laboratory-evolved mutant of CrtI (four- to six-step) (180); CrtO, β,β-carotene 4,(4′)-ketolase; CrtU, β,β-carotene desaturase; CrtX, zeaxanthin glycosylase; CrtY, lycopene β-cyclase; $CrtY_2$, laboratory-evolved mutant of CrtY (180); CrtZ, β,β-carotene 3,(3′)-hydroxylase. Biosynthesis of oxygenated derivatives of ζ-carotene and neurosporene was attributed to early termination by CrtA of the desaturation sequence of CrtI, which is normally a four-step desaturase (118). Although CrtA is primarily a ketolase, it is thought to catalyze the introduction of both hydroxy groups of phillipsiaxanthin (C. Schmidt-Dannert, personal communication). This figure was made using data from reference 118.

Although lycopene, with its two ψ-ends, is the natural substrate for most known cyclases, studies have shown that some cyclases can act on a wider range of substrates (Fig. 8b). An ability to convert different substrates was reported for *Arabidopsis* ε-end lycopene cyclase, which can cyclize the ψ-end of neurosporene to form α-zeacarotene (53, 54). Britton noted that the only apparent requirement for recognition and catalysis by carotenoid cyclases is that the substrate have a ψ-end (Fig. 8b) (32). However, Takaichi et al. showed that bacterial and plant lycopene cyclases can cyclize the 7,8-dihydro-ψ-end of ζ-carotene and neurosporene (199). Recently, we showed that *E. uredovora* β-cyclase and a plant ε-cyclase can efficiently cyclize nonnatural carotenoids with a C_{35} backbone (211). Similarly, Lee et al. reported that the C_{30} carotenoid 4,4′-diaponeurosporene can be cyclized by *Erwinia* β-cyclases, leading to the novel cyclic C_{30} carotenoid diapotorulene (118). Another interesting enzyme is capsanthin-capsorubin synthase (CCS) from *Capsicum annuum*. The primary natural function of CCS is to rearrange the epoxidized cyclic carotenoids antheraxanthin and violaxanthin into the cyclopentyl κ-end products capsanthin and capsorubin. When expressed in *E. coli*, however, CCS was shown to also possess lycopene β-cyclase activity, cyclizing both ends of lycopene to yield β,β-carotene (84).

All known β-cyclases, except one recently discovered in a marine bacterium (207), create rings at both ends of lycopene. In contrast, known ε-cyclases except those from *Lactuca sativa* (lettuce) and the flower *Adonis aestivalis* generate monocyclic carotenoids (52). In an attempt to manipulate the ring number and understand how it is determined, Cunningham and Gantt constructed a series of single-crossover chimeras of one-step (*Arabidopsis thaliana*) and two-step (*Lactuca sativa*) ε-cyclases (52). Analysis revealed a region of six amino acids involved in ring number determination. Further mutagenesis experiments identified single-amino-acid substitutions that alter ring number specificity: in the lettuce two-step cyclase Dy4, substitution of H457 with leucine converted the enzyme into a monocy-

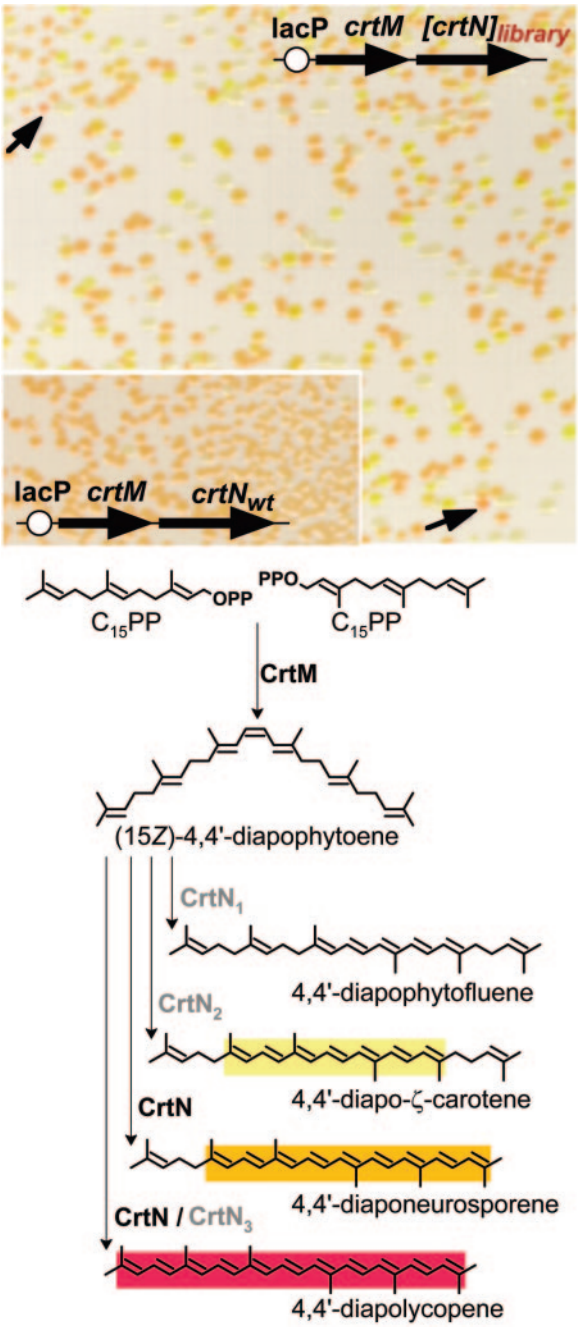


FIG. 7. The step number of the C_{30} desaturase CrtN is readily modified by mutation. A library of mutant *crtN* alleles was coexpressed with the C_{30} carotenoid synthase *crtM* in *E. coli.* Wild-type CrtN is a three- to four-step desaturase when expressed in *E. coli*, causing colonies to appear orange (inset). Colonies harboring CrtN mutants with increased step number were deep orange and red (highlighted by arrows), reflecting the longer carotenoid chromophores formed by these mutant desaturases. Colonies harboring mutants with decreased step number appear yellow/lemon, while colonies with nonfunctional or single-step desaturases are pale since these products are colorless. Shown below are the possible C_{30} desaturation products and the desaturases that synthesize them. $CrtN_{1-3}$ are discovered mutants of CrtN with altered desaturation step number. Each colored box depicts the approximate color of the carotenoid in white light. Molecules without colored boxes are colorless. Abbreviations: lacP, *lac* promoter; wt, wild type.

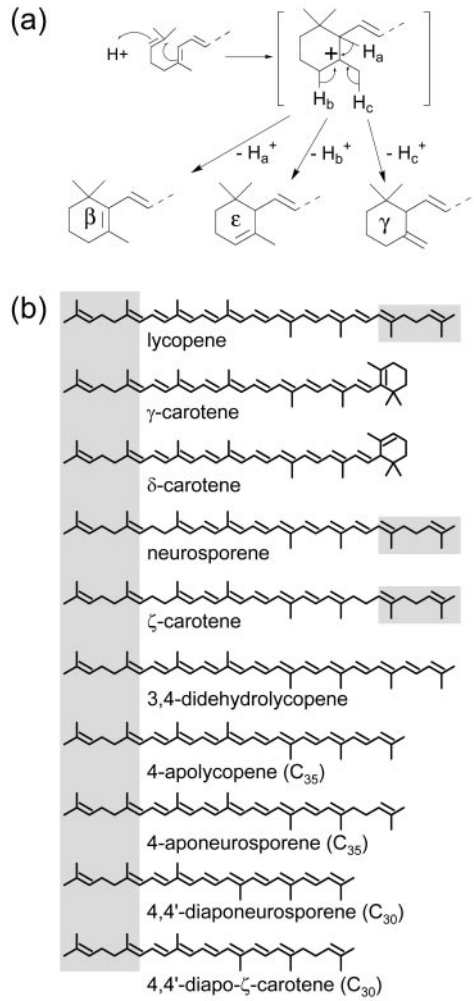


FIG. 8. Cyclization of carotenoids. (a) Mechanism of carotenoid cyclization. Lycopene with its two ψ-end groups is the preferred substrate for all known carotenoid cyclases. All cyclases produce the same carbocationic intermediate on protonation of C-2 of the substrate. Product ring type (β, ε, or γ) is determined by which proton (a, b, or c in the figure) is eliminated in the subsequent rearrangement step. This panel was made using data from reference 32. (b) Substrates accepted by lycopene cyclases. The shaded portion(s) of each structure is cyclized. The first three compounds, with their ψ-end groups, have long been known as natural substrates for lycopene cyclases. The remaining seven structures, including neurosporene with its 7,8-dihydro-ψ-end (left side of molecule) and ζ-carotene with two 7,8-dihydro-ψ-ends, have more recently been shown to be cyclized.

clase; the corresponding L448H mutation in Y2, the *Arabidopsis* monocyclase, resulted in an enzyme that forms two rings. Thus, ring number specificity can be modulated with a single amino acid substitution. The authors of this study suggested that the residues they identified may play a role in dimer formation (52). Lycopene may be oriented in the plasma membrane such that one of its ends is more accessible to the cyclase than the other. Only when cyclase dimers are formed does binding of the more accessible end bring the less accessible end close enough to the other subunit for it to be cyclized as well. An alternative but similar explanation for the above observations is that the mutations alter the cyclase oligomerization state within a carotenoid-synthesizing complex. For example,

when one cyclase subunit is present in each complex, carotenoid products are cyclized on only one end whereas complexes with two cyclase subunits would cyclize carotenoids at both ends. Association of cyclase monomers in a complex would depend on their interactions with each other and the other constituents of the complex, and a single amino acid substitution could be sufficient to disrupt (or promote) this association. It is also possible that the single amino acid substitutions described above alter the enzyme's intrinsic preference for the carotenoid substrate that has already been cyclized on one end. The H457L mutation may reduce the preference of Dy4 for the monocyclic substrate, while the L448H mutation increases the ability of Y2 to cyclize it.

$CrtI_{14}$ is a four- to six-step desaturase, and *E. coli* cells expressing this variant along with CrtE (a C_{20}PP synthase) and CrtB (a C_{40} carotenoid synthase) can synthesize 3,4,3′,4′-tetradehydrolycopene (Fig. 6) (181). When CrtY, the β-cyclase from *Erwinia*, was coexpressed with these enzymes, the cells synthesized exclusively β,β-carotene—the same product made by cells expressing wild-type CrtE, CrtB, CrtI, and CrtY. Directed evolution, however, could create a pathway leading to a new cyclized product: after performing DNA shuffling to make a library of CrtY variants and coexpressing these with $CrtI_{14}$ and CrtE, Schmidt-Dannert et al. identified clones that accumulated the monocyclic carotenoid torulene (Fig. 6) (181). Pigment analysis from cells harboring the CrtY mutants including $CrtY_2$ (Fig. 6) revealed torulene together with lycopene, 3,4,3′,4′-tetradehydrolycopene, β,ψ-carotene, and β,β-carotene. Torulene-producing variants were also discovered when the CrtY library was created by error-prone PCR (Umeno, unpublished). These mutants made up 2 to 5% of the library. This high frequency of torulene-producing mutants suggests that the torulene pathway emerged by down-regulation of cyclase activity. Mutants of CrtY with decreased catalytic activity would compete less efficiently with $CrtI_{14}$ for the ends of the carotenoid substrate, with the result that only one end is cyclized while the other is desaturated, leading to torulene. Alternatively, the CrtY mutants found in the torulene-producing clones may be compromised in their ability to form dimers, either as part of larger carotenoid enzyme complexes or not.

Although the underlying mechanisms for the changes in cyclase step number remain obscure, it is clear that carotenoid cyclases are evolvable: their phenotype can change dramatically with a small number of mutations, and there appear to be multiple pathways to a particular phenotype. As we show in the next section, combining evolved carotenoid desaturases and cyclases with locally specific enzymes further downstream allows an even larger natural product space to be sampled. To date, carotenoid cyclases have not been evolved in the laboratory for altered product specificity, for example to convert a β-cyclase to an ε-cyclase or vice versa. These cyclases have very similar chemical mechanisms (Fig. 8a), and we predict that cyclase product specificity will be easily modified by mutation.

Enzymes catalyzing further modifications. Numerous enzymes catalyzing hydroxylation, epoxidation, glycosylation, O methylation, acyl transfer, prenyl transfer, oxidative cleavage, and other reactions on cyclic and acyclic carotenoids have been cloned and expressed in *E. coli* (for reviews, see references 175 and 180). Misawa et al. made an early demonstration of the localized specificity of these modifying enzymes (135). Introduction of the β-end 3-hydroxylase CrtZ and the β-end 4-ketolase CrtW into *E. coli* harboring a β,β-carotene pathway led to the accumulation of as many as nine carotenoids (Fig. 3). Recent work has revealed that many carotenoid-modifying enzymes act on a range of substrates (5, 16, 118, 191, 197).

Lee et al. exploited the catalytic promiscuity of a number of carotenoid-modifying enzymes in an *E. coli* system to extend laboratory-evolved pathways to 3,4,3′,4′-tetradehydrolycopene and torulene, generating biosynthetic routes to several carotenoids not identified in nature (118) (Fig. 6). CrtA, an oxygenase from *Rhodobacter capsulatus* that normally acts on the methoxy carotenoid spheroidene, was found to insert one keto group into ζ-carotene, neurosporene, and lycopene and to insert two keto groups into tetradehydrolycopene. Monocyclic torulene, synthesized via the pathway constructed using the laboratory-evolved desaturase and cyclase (181), served as a substrate for CrtO, a β,β-carotene ketolase from *Synechocystis* sp. strain PCC 6803; CrtU, a β,β-carotene ring desaturase from *Brevibacterium linens*; and CrtZ, a β,β-carotene hydroxylase from *E. herbicola*. In addition, 3-hydroxytorulene, the product of the action of CrtZ on torulene, was further metabolized by CrtX, a zeaxanthin glycosylase from *E. herbicola*, leading to the synthesis of torulene-3-β-D-glucoside (Fig. 6). In this work, the pathway to torulene generated by directed evolution of two upstream carotenoid biosynthetic enzymes was extended in several different directions by adding genes for further transformations. Owing to their intrinsic localized specificity, coexpression of these downstream modifying enzymes with the foreign torulene pathway resulted in a series of novel torulene derivatives.

There are no reports yet of directed evolution of carotenoid oxygenases or post-cyclase carotenoid-modifying enzymes. Work by Sun et al. (197), however, provides an interesting example of how modifying the sequence of a carotenoid hydroxylase can alter the enzyme's product distribution. The β,β-carotene hydroxylase from *A. thaliana* catalyzes two hydroxylation steps, converting β,β-carotene to zeaxanthin (>90%) in *A. thaliana* and when expressed in *E. coli* (Fig. 9). Noticing that this enzyme had an N-terminal extension of more than 130 amino acids compared with other known β,β-carotene hydroxylases, Sun et al. expressed in *E. coli* a truncated version of the *Arabidopsis* hydroxylase lacking the N-terminal 129 amino acids. The truncated enzyme primarily catalyzed only one hydroxylation step, and β-cryptoxanthin (Fig. 9) accumulated as the main product (>75%) (197). The molecular basis for this altered hydroxylation step number is not known; the authors speculated that truncation yielded an enzyme deficient in the ability to form dimers.

Carotenoid cleavage enzymes comprise an important class of carotenoid-modifying enzymes. Enzymatic oxidative cleavage of carotenoids in both carotenogenic and non carotenogenic organisms creates diverse "apocarotenoid" structures, many of which are highly biologically significant (71). Well known products of carotenoid cleavage enzymes include retinol and retinoic acids (mammalian hormones) (220), abscisic acid (a plant growth regulator) (186, 236), and the plant pigment bixin (28). The localized specificity of several carotenoid cleavage enzymes has recently been demonstrated. One important cleavage enzyme is β,β-carotene-15,15′-monooxygenase (βCM) (formerly named β,β-carotene-15,15′-dioxygenase be-

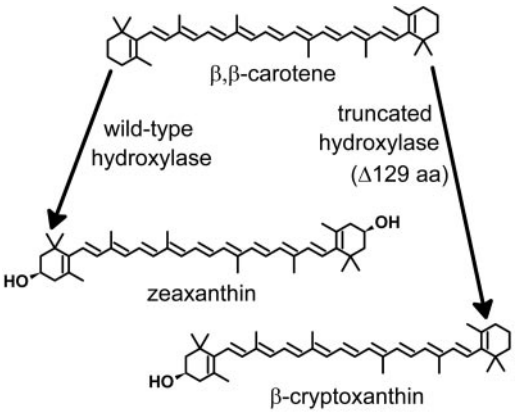


FIG. 9. Alteration of product specificity of the β,β-carotene hydroxylase from *Arabidopsis thaliana.* The wild-type enzyme catalyzes two hydroxylation steps, converting β,β-carotene to zeaxanthin when expressed in either *A. thaliana* or *E. coli.* When the N-terminal 129 amino acids (aa) were removed, the truncated enzyme expressed in *E. coli* catalyzed only one hydroxylation step, leading primarily to synthesis of β-cryptoxanthin (197).

fore the enzyme was shown to employ a monooxygenase mechanism [121]), which converts β,β-carotene into retinal (Fig. 10a) (158, 165, 219, 231, 232). βCM cleaves substrates other than β,β-carotene, such as α-carotene (228) and β-cryptoxanthin (Fig. 10a) (123, 228). The enzyme has a strong preference for substrates with at least one unsubstituted β-end (123, 228) and appears to always cleave a substrate's central 15-15′ double bond (115). Recently discovered homologs of βCM catalyze the asymmetric cleavage of carotenoids (28, 98, 186). One murine oxygenase cleaves β,β-carotene at the 9′-10′ double bond (Fig. 10b), yielding β-apo-10′-carotenal (C_{27}) and β-ionone (C_{13}) (98). This enzyme also catalyzes the cleavage of lycopene, resulting in the formation of apolycopenals (Fig. 10b) (98). Another carotenoid cleavage enzyme from *A. thaliana* was shown to cleave an impressive array of different substrates in vitro (Fig. 10c) (185). Thus, carotenoid cleavage enzymes are quite versatile and their localized specificity may be exploited to generate diverse new carotenoid derivatives in the laboratory.

Creation of Pathways to Carotenoids with New Carbon Scaffolds

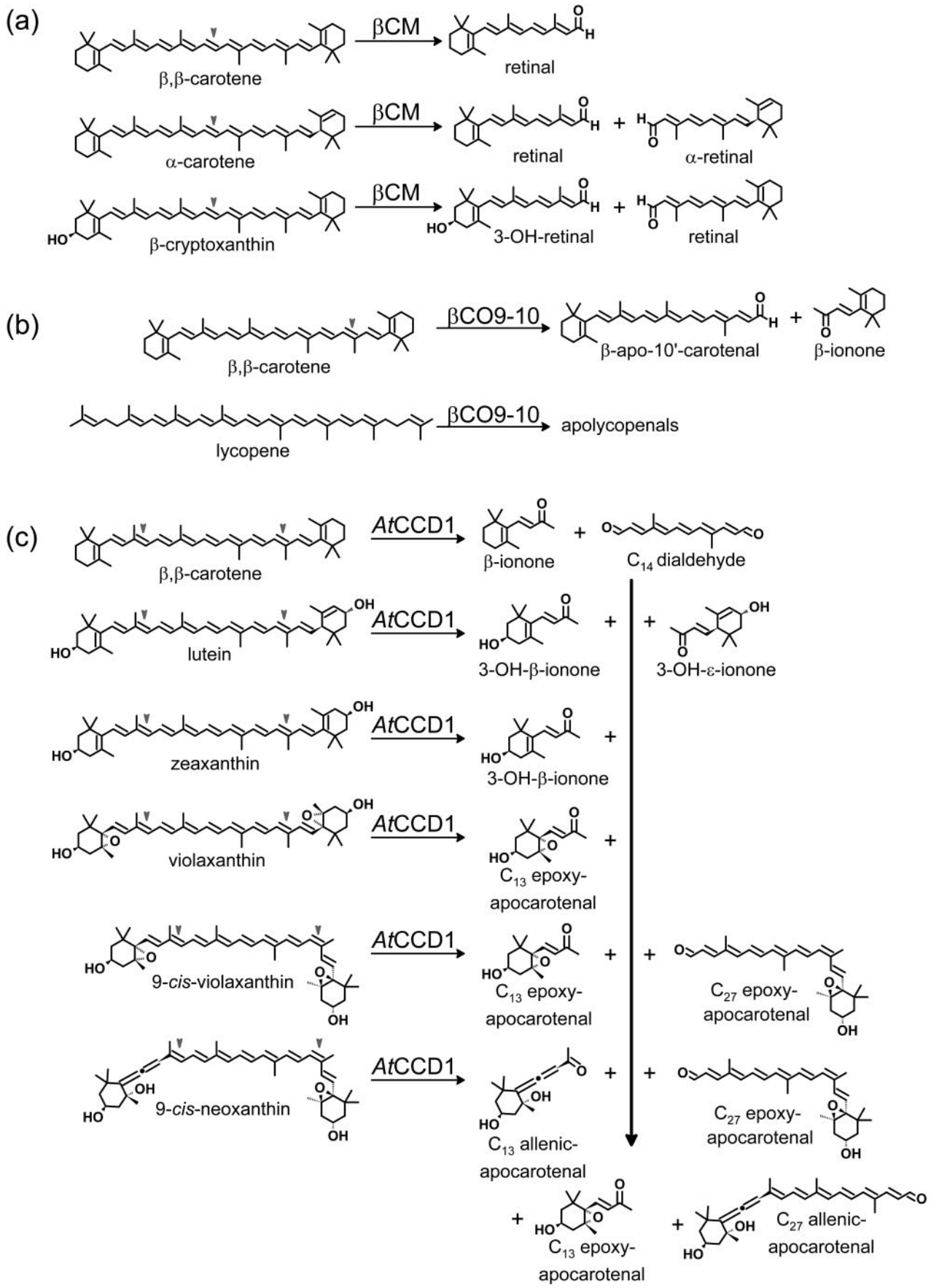
Given the proven evolvability of different enzymes involved in carotenoid biosynthesis, we investigated the possibility of creating routes to whole new families of carotenoids built on scaffolds other than the symmetric C_{30} and C_{40} backbones seen in nature. We reasoned that if carotenoid synthases could be engineered to accept different substrates and synthesize unnatural carotenoid backbones, locally specific downstream enzymes might accept and modify these backbones, leading to whole new families of carotenoids. The downstream enzymes could also be evolved to produce still more novel products or to function more efficiently on their new substrates. Compared to increasing the diversity of known C_{30} or C_{40} carotenoids by altering the specificity of downstream pathway enzymes, generating entire new pathway families by focusing on upstream enzymes has a much greater capability to explore new carotenoid structures. The entire space of carotenoids built on non-C_{30} or non-C_{40} backbones, for example, is uncharted. Generating novel chain length carotenoids can also be viewed as a rigorous test of carotenoid pathway evolvability.

A particularly exciting goal is to generate carotenoids with chromophores or physico-chemical properties not possible in carotenoids based on natural backbones. Millions of years of evolution gave rise to the ~700 carotenoids known in nature. Laboratory evolution of new carotenoid pathways based on unnatural backbones has the potential to at least double or triple this diversity. If significant numbers of new products can be generated, laboratory pathway evolution would be validated as an important tool for preparing large natural product libraries from which compounds with valuable properties and biological activities can be discovered.

A C_{35} carotenoid pathway. We recently discovered that the C_{30} synthase CrtM could assemble a 35-carbon backbone when supplied with C_{15}PP and C_{20}PP precursors (211). When transformed with *crtE* from *E. uredovora* (encoding a C_{20}PP synthase) and *crtM* from *S. aureus*, *E. coli* cells accumulate 4-apophytoene, an asymmetrical C_{35} carotenoid formed via heterocondensation of C_{15}PP and C_{20}PP (Fig. 11). This novel backbone comprised 40 to 60% of total carotenoids, with C_{30} 4,4′-diapophytoene and C_{40} phytoene as minor components.

We found that the C_{35} backbone was further metabolized by various C_{40}- and C_{30}-carotenoid biosynthetic enzymes, creating a family of new C_{35} carotenoids (Fig. 11). Carotenoid desaturases from C_{40} (*Erwinia* CrtI) or C_{30} (*Staphylococcus* CrtN) pathways efficiently converted the C_{35} backbone to yield acyclic carotenoids with different levels of desaturation (211). It is interesting that the C_{30} desaturase CrtN showed higher step number (four or five steps) in the C_{35} pathway than in its native C_{30} pathway (three or four steps) or in a C_{40} pathway (two or three steps). Thus, *E. coli* cells expressing CrtN develop an intense red color only when they produce the C_{35} substrate. The larger number of desaturation steps catalyzed on a C_{35} substrate may reflect a tradeoff between the enzyme's substrate preference and the number of desaturation steps the substrate can possibly undergo. CrtN most probably prefers its native C_{30} substrate, but the C_{40} substrate can undergo more desaturations. With a C_{35} substrate, a balance of these two factors can yield the high observed step number if, for example, the enzyme (or enzyme complex) binds the "C_{30}-like" end of the C_{35} substrate strongly and catalyzes one or more additional desaturation steps on the "C_{40}-like" end compared with a C_{30} sub-

FIG. 10. Several carotenoid cleavage enzymes display localized specificity. Gray arrowheads show bonds cleaved. (a) β,β-Carotene-15,15′-monooxygenase (βCM) can cleave substrates with an unsubstituted β-end (123, 228). (b) Murine β,β-carotene-9′,10′-oxygenase (βCO9-10) can also cleave lycopene, leading to uncharacterized apolycopenals (98). (c) One carotenoid cleavage dioxygenase from *A. thaliana* (*At*CCD1) cleaves at least six different carotenoid substrates, leading to a variety of products (185). The downward arrow from C_{14} dialdehyde indicates that this product was detected from all six substrates shown.


(a)
βCM
β,β-carotene
retinal
α-carotene
α-retinal
β-cryptoxanthin
3-OH-retinal
(b)
βCO9-10
β-apo-10'-carotenal
β-ionone
lycopene
apolycopenals
(c)
AtCCD1
lutein
3-OH-β-ionone
3-OH-ε-ionone
zeaxanthin
violaxanthin
C13 epoxy-apocarotenal
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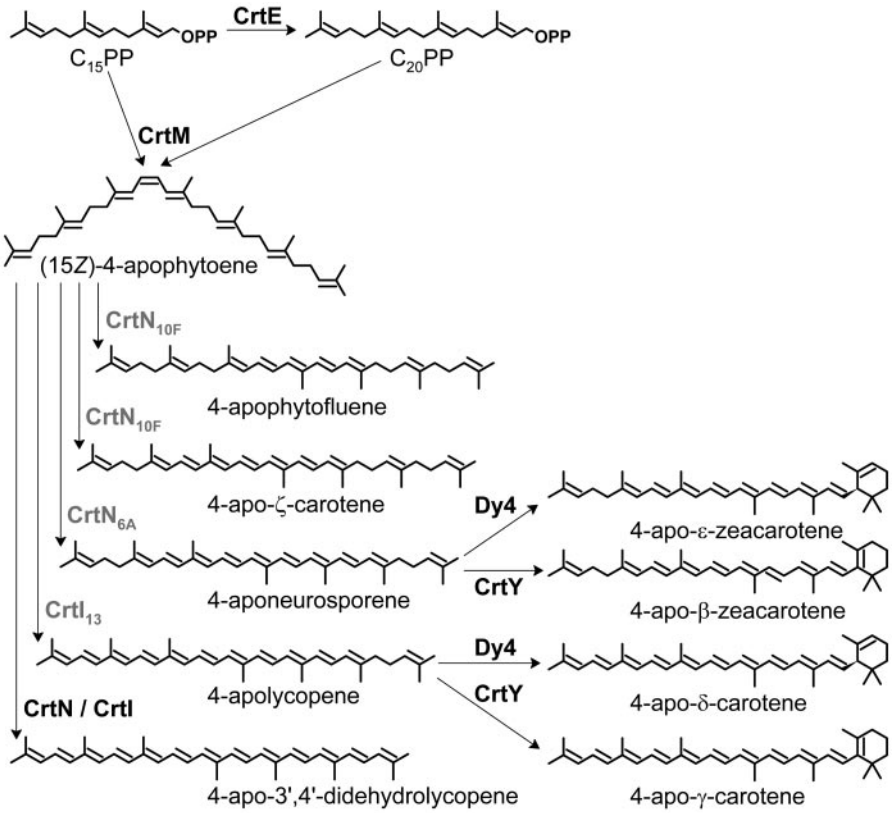

FIG. 11. Generation of a C_{35} carotenoid pathway by gene assembly and directed enzyme evolution (211). When supplied with $C_{15}PP$ and $C_{20}PP$, the C_{30} carotenoid synthase CrtM produces the C_{35} carotenoid backbone, 4-apophytoene. By coexpressing C_{30} or C_{40} desaturases and mutants thereof (gray), different clones could be found that produce each possible acyclic C_{35} carotenoid as the main product. *E. uredovora* (*P. ananatis*) β-cyclase (CrtY) and *L. sativa* ε-cyclase (Dy4) were shown to cyclize two of the C_{35} substrates, leading to four different cyclic C_{35} carotenoids. All 10 C_{35} carotenoids in this figure had never been previously reported.

strate. Other scenarios can also plausibly lead to the same result.

Assembling the enzymes for the new backbone (CrtE and CrtM) with wild-type desaturases provided some of the possible C_{35} desaturation products. The remaining desaturation products were obtained by directed evolution. Random mutagenesis of carotenoid desaturases expressed in *E. coli* synthesizing the C_{35} carotenoid backbone generated colonies having a spectrum of colors, which reflected desaturase mutants with altered step numbers (211). In fact, individual clones that produced each of the possible acyclic C_{35} carotenoids as the main product were identified (Fig. 11). Addition of the β-cyclase CrtY from *E. uredovora* or the ε-cyclase Dy4 (*L. sativa*) further diversified the C_{35} pathway, yielding at least four new monocyclic C_{35} carotenoids (Fig. 11).

As discussed in the Introduction, some fungi naturally accumulate carotenoids with 35 carbon atoms (12, 14, 164, 215). All of these are monocyclic oxygenated carotenoids (xanthophylls) and are believed to result from the action of an unidentified oxidative cleavage enzyme on C_{40} carotenoids such as torulene. Disruption of cyclase activity in *Neurospora crassa* resulted in the biosynthesis of an acyclic C_{35} xanthophyll (12). These C_{35} xanthophylls and the biosynthetic routes leading to their formation are qualitatively different from laboratory-generated C_{35} carotenoids biosynthesized via heterocondensation of $C_{15}PP$ and $C_{20}PP$. Notably, the direct route to the C_{35} carotenoid backbone 4-apophytoene opens many possibilities for further pathway diversification by downstream enzymes (desaturases, cyclases, etc.) whereas many fewer options exist for diversifying the already desaturated, cyclized, and oxygenated C_{35} carotenoids found in fungi.

Because C_{35} carotenoids have asymmetric backbones, each desaturation step can potentially yield more than one product, depending on the direction of the desaturation step (and the previous steps). Thus, acyclic C_{35} carotenoids with 3, 5, 7, 9, 11, and 13 conjugated double bonds can assume 1, 2, 3, 3, 2, and 1 (a total of 12) possible structures, respectively. In this sense, the C_{35} pathway is inherently more complex and explores a much larger "structure space" than do pathways based on the symmetric C_{30} and C_{40} carotenoid backbones. Cyclization and other modifications of the backbone can further increase the number of possible C_{35} carotenoids. Considering their broad substrate tolerances, we expect that other carotenoid-modifying enzymes will also be functional in this C_{35} pathway and that one could make many hundreds of new carotenoids. The generation of a new, full-fledged C_{35} carotenoid biosynthetic pathway in the laboratory is a convincing demonstration of the evolvable nature of carotenoid biosynthetic enzymes and pathways and serves as an excellent illustration of how gene assembly coupled with directed evolution can rapidly access diverse chemical structures.

Carotenoids with longer backbones. The C_{25}PP isoprenoid precursor farnesylgeranyl diphosphate and the C_{30}PP hexaprenyl diphosphate serve as components of archaebacterial membrane lipids and quinone side chains, respectively. They have never been found to be incorporated into carotenoids. However, these longer analogs of the C_{40} carotenoid precursor C_{20}PP could in principle be used in the biosynthesis of carotenoid backbones having 45, 50, 55, or 60 carbon atoms. Similarly, the monoterpene precursor geranyl diphosphate (C_{10}PP) could, in theory, be used to construct C_{20}, C_{25}, or asymmetric C_{30} carotenoids. All these backbone structures would be possible, provided that there were synthases capable of condensing these substrates. Although carotenoid synthases with these activities are not known in nature, we reasoned that an existing synthase could be engineered to make at least some of these new backbones.

As discussed above, we had probed carotenoid synthase evolvability by screening for mutants of CrtM that function as a C_{40} synthase and mutants of CrtB that function as a C_{30} synthase. CrtM and CrtB diverged long ago and have only 30% amino acid sequence identity. Nonetheless, CrtM was readily converted to a C_{40} synthase (214). Sequencing confirmed that multiple single-substitution pathways leading to this phenotype were possible (213). We then tested whether one or more of these CrtM mutants might be able to synthesize even larger backbones.

To supply a C_{25}PP substrate in *E. coli*, we constructed and expressed the Y81A variant of the C_{15}PP synthase from *B. stearothermophilus* ($BstFPS_{Y81A}$). This variant was shown by Ohnuma's group to produce C_{25}PP as the main product in vitro (150, 151). When coexpressed with $BstFPS_{Y81A}$, the single mutants $CrtM_{F26L}$ and $CrtM_{F26S}$ discovered in our original C_{40} pathway screening experiment (214) synthesized 16-isopentenylphytoene (the C_{45} carotenoid backbone, C_{20} + C_{25}) and 16,16′-diisopentenylphytoene (the C_{50} backbone, C_{25} + C_{25}) (Fig. 12a). Site saturation mutagenesis at residues 26, 38, and 180 (also discovered to play a role in conferring C_{40} function on CrtM [213]) resulted in several double and triple mutants with improved ability to synthesize C_{45} and C_{50} carotenoid backbones (212). These carotenoid backbones have never been reported in nature.

Interestingly, some of the new CrtM-based C_{45} and C_{50} synthases showed almost no activity in the original C_{30} pathway (212). Thus, carotenoid synthases, although highly substrate-specific in their natural pathways, can both broaden and shift specificity with minimal genetic change. We are interested in finding whether these variants can be engineered further to become producers of C_{50} carotenoids exclusively (i.e., no smaller side products) or to generate even larger carotenoids, with 60- or even 70-carbon chains. Although such long structures may not be biologically relevant (and may even be toxic), we might be able to make them in an engineered pathway in *E. coli*.

When transformed with *crtI* (phytoene desaturase), *E. coli* harboring the C_{45}-C_{50} pathway accumulated a deep red color not seen when CrtI is expressed in cells producing only C_{40} carotenoids (Fig. 12b). Further analysis revealed that the C_{45} backbone is completely metabolized by CrtI, although residual, undesaturated C_{50} carotenoid backbones have been detected in some cell extracts (A. Tobias, unpublished data). Given the proven evolvability of carotene desaturases and cyclases and the localized specificity of the carotenoid-modifying enzymes further downstream, we envision that it will be possible to generate many new carotenoids based on these backbones.

Whereas the fully desaturated, red C_{40} carotenoid 3,4,3′,4′-tetradehydrolycopene has a chromophore consisting of 15 conjugated double bonds, C_{45} and C_{50} carotenoid backbones can accommodate 17 and 19, respectively. Extension of a conjugated system increases the wavelength(s) of light absorbed by a molecule and predictably changes the molecule's color as observed under white light (33). Dodecapreno-β-carotene, a chemically synthesized analog of β,β-carotene with 19 conjugated double bonds, was reported to absorb light at wavelengths longer than 600 nm and to possess bordeaux red to blue-violet pigmentation, depending on the solvent (93). Two of this molecule's conjugated double bonds are contributed by the two sterically-hindered β-ionone groups. Therefore, just as lycopene with 11 all-*trans*-conjugated double bonds absorbs longer-wavelength light than β,β-carotene with 9 *trans* plus 2 beta double bonds, a linear, all-*trans* carotenoid with 19 conjugated double bonds would absorb even longer-wavelength light and would possess even further blue-shifted pigmentation than dodecapreno-β-carotene. Carotenoids with 19 all-*trans* double bonds would have possible uses as colorants and might possess interesting or unique antioxidant properties.

DISCUSSION AND FUTURE DIRECTIONS

Revised View of Carotenoid Biosynthetic Pathways

Carotenoid biosynthetic pathways have been described as having a tree-like organization, as depicted in Fig. 1 (8). The conserved upstream reactions leading to backbone synthesis form the trunk, which supports many branches (and subbranches) representing increasingly diverse and species-specific downstream steps. However, many examples outlined in this review have shown that enzymes from one branch can often convert substrates from a different branch. Thus, in light of the ease with which it is now possible to combine carotenoid biosynthetic genes from diverse sources in recombinant organisms, pathway engineers might be better served by replacing the carotenoid "tree" with a more web-like model that emphasizes the interconnectivity of enzymes and metabolites and deemphasizes the natural circumstances in which they are found. In addition, the tree model, with its conserved trunk and variable branches, implies that the major mode of diversity generation is through the addition of new branches and subbranches. However, we now know that an important source of new carotenoid diversity is the generation of new trunks (new backbones), which can quickly be filled with many branches of their own by using enzymes taken from nature's extant pathway branches and evolving them in their new pathway context.

Future Challenge: Specific Pathways

To date, most laboratory-evolved and many engineered pathways accumulate novel carotenoids as components of a complex mixture. This may be fine and sufficient for the discovery of new compounds, but to be practically useful as a synthetic route to a specific compound, for example, the path-

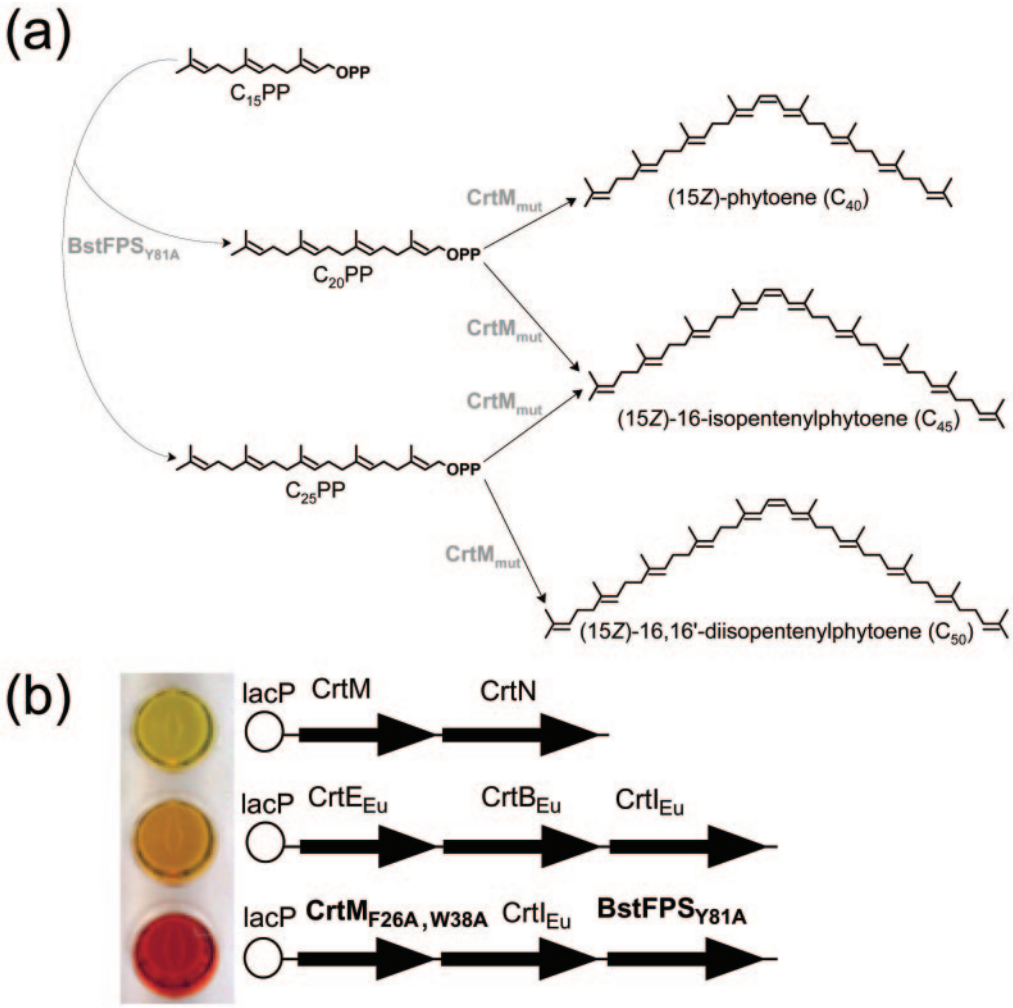

FIG. 12. Biosynthesis of carotenoids with C_{45} and C_{50} backbones (212). (a) Expression of the Y81A variant of the C_{15}PP synthase from *B. stearothermophilus* ($BstFPS_{Y81A}$) led to the production of C_{20}PP and C_{25}PP in *E. coli.* Additional coexpression of variants of the C_{30} carotenoid synthase CrtM mutated at F26 and/or W38 ($CrtM_{mut}$) resulted in biosynthesis of the C_{45} carotenoid backbone 16-isopentenylphytoene ($C_{20} + C_{25}$) and the C_{50} backbone 16,16′-diisopentenylphytoene ($C_{25} + C_{25}$). Both of these larger carotenoids had never been previously reported. (b) *E. coli* cells expressing $BstFPS_{Y81A}$, $CrtM_{F26A,W38A}$, and the C_{40} desaturase CrtI accumulated as yet uncharacterized desaturated carotenoids with a deep red color (third row). Shown for color comparison is the acetone extract of an *E. coli* culture expressing CrtM and CrtN and synthesizing the C_{30} carotenoids 4,4′-diaponeurosporene and 4,4′-diapolycopene (first row) and the acetone extract of an *E. coli* culture expressing CrtE, CrtB, and CrtI and synthesizing the C_{40} carotenoids lycopene and 3,4,3′,4′-tetradehydrolycopene (second row). Abbreviations: lacP, *lac* promoter; Eu, *Erwinia uredovora* (current approved name, *Pantoea ananatis*).

way must undergo further engineering to become more specific. Natural carotenoid pathways, which tend to produce only a small number of end products, probably followed a similar course of "pruning" many products down to a chosen few. As laboratory evolution experiments demonstrate, invention is an overnight event but shaping newly discovered pathways into mature, specific ones can require much subsequent engineering effort. In the laboratory thus far, we have succeeded only in the first step, and real work remains. We list several possible approaches to converting a newborn, nonspecific pathway into one that makes a single novel metabolite.

Evolving enzyme specificity. The most straightforward (but maybe the most labor-intensive) way to control pathway specificity is to engineer more specific biosynthetic enzymes. Most laboratory-evolved enzymes, however, show broadened rather than shifted specificity, and therefore the pathways containing them accumulate a combination of old and new products. In contrast, nature often does a perfect job, such that two related enzymes (with a common ancestor but now sitting in different pathways) show no overlap in their substrate or product ranges.

We think that pathways specific for newly discovered metabolites can be engineered, possibly after accumulating neutral mutations (in the context of the desired pathway) and certainly by directly screening for mutants that produce less of the old compounds while maintaining (or even improving) their ability to synthesize the new ones. The laboratory-discovered C_{35}, C_{45}, and C_{50} carotenoid pathways provide sophisticated experimental systems for testing this approach to dealing with what is a central, and challenging, problem in metabolic-pathway engineering. In addition, such experiments may contribute to our understanding of the early evolution of metabolism. The increasingly favored "patchwork" model of pathway

evolution states that the specific metabolic pathways we observe today evolved from leaky assemblages of nonspecific enzymes (90, 171, 233) but provides no molecular explanation for how this happened. Experiments aimed at evolving pathway specificity in the laboratory would permit researchers to track the impact of each genetic change as the pathways acquire greater specificity, and they should therefore provide molecular insight into this poorly understood process.

Designed channeling. Channeling of intermediates between successive enzymes in a pathway can eliminate undesirable fluxes and byproducts. As mentioned above, there is evidence supporting the existence of carotenoid biosynthetic enzyme complexes, although we do not know what role such complexes might play in pathways assembled in a recombinant organism. Designing enzyme complexes that can channel or shuttle intermediates from one enzyme or catalytic site to the next in a particular sequence may find a use in directing the pathway toward efficient production of desired metabolites. Nature discovered this strategy for other metabolites, giving rise to the marvelous enzymatic assembly lines that synthesize polyketides and nonribosomal peptides (reviewed in reference 169).

In an effort to engineer substrate channeling between successive enzymes in a biosynthetic pathway, Brodelius et al. fused a C_{15}PP synthase with a sesquiterpene cyclase (34). The resultant fusion enzyme was more efficient at converting isopentenyl diphosphate into the cyclized C_{15} product than was the corresponding quantity of mixed unfused enzymes (34). Efforts in this direction might better regulate recombinant carotenoid pathways in *E. coli*, although significant structural information, which is currently unavailable for carotenoid enzymes, may be required.

Specificity by flux balance. Even pathways harboring individually promiscuous enzymes can achieve reasonable specificity as a whole if all steps are properly balanced. If the activity or expression level of each enzyme is properly tuned, production of the desired product can be enhanced and the proportion of unwanted side products can be diminished. In addition to modifying the sequences of promoter or ribosomal binding sites, Smolke et al. have shown that the individual expression levels of multiple enzymes can be tuned to some degree by altering the transcriptional order of the genes in an operon and varying the stability of mRNA toward nuclease degradation by using engineered hairpin structures or RNase recognition sites (189). Although adjusting the expression levels of enzymes with broad substrate or product specificity is unlikely to eliminate undesired metabolites outright, we have observed that significant increases in the ratio of new to old pathway products can be realized from this approach (unpublished data).

Specificity by diversion of unwanted intermediates. Balancing enzyme activities or expression levels cannot eliminate side products if an enzyme converts a single substrate into multiple products, only one of which is desired. If such enzymes cannot be engineered to narrow their product range, one strategy for achieving pathway specificity is to selectively divert unwanted intermediates away from downstream enzymes in the pathway. Instead, these intermediates could be catabolized or incorporated into products that are easily separated from the desired one(s) by introducing appropriately selective enzymes. In terms of the conversion yield of precursors into products, this approach is more wasteful than is engineering enzymes to be more specific. However, because product recovery costs can vastly exceed fermentation expenses in industrial processes, product purity can be more important than product titer (23). Thus, if a tradeoff is unavoidable, the better choice may be to accept lower product yield in favor of increased purity.

Prospects and Challenges for Diversifying Other Pathways by Directed Evolution

Although the best examples of diversifying metabolic pathways by directed evolution come from work on the carotenoids, this approach can be applied to other natural product pathways as well. There are very good indications that other pathways and biosynthetic enzymes will be as readily evolvable in the laboratory as the carotenoid pathways. Jones and Firn hypothesize that many natural product pathways are in fact already conducting their own combinatorial biosynthesis and high-throughput screening programs, at least on evolutionary timescales (62–64, 91). Because the probability that any particular small molecule will have potent biological activity is inherently low, Jones and Firn argue that organisms increase the odds of discovering new metabolites that provide a selective advantage by exploring a large number of different structures. Thus, the argument continues, natural product pathways have evolved traits that maximize the production and retention of product diversity while minimizing the costs of synthesizing different metabolites, most of which are not beneficial to the producer. Among these traits are branched pathway structures, which enable the discovery of new metabolites as well as the retention of existing ones; matrix pathways or metabolic grids, where a small number of enzymes convert structurally similar precursors to a large number of products by multiple routes; and promiscuous enzymes, which can accept a variety of substrates and convert newly discovered metabolites into additional new products. As we have shown, carotenoid pathways possess these traits as well as others (e.g., individual enzymes are evolvable) that can be exploited for forward evolution. On the other hand, because carotenoids already fulfill important biological roles and no organism is known to synthesize a large number of different carotenoids, it is not apparent that nature is conducting massive searches for new and improved carotenoids. Other natural product pathways, however, do appear to be in "search" mode (for example, the bracken fern makes at least 27 different sesquiterpene indan-1-ones [91]), and these pathways may be even more evolvable than carotenoid pathways.

Isoprenoid pathways are an obvious target for laboratory evolution. Many isoprenoid biosynthetic enzymes accept a variety of natural and unnatural substrates (91, 139, 140, 147). The gibberellins, a class of diterpenoid hormones with more than 120 known members, are synthesized by a metabolic grid composed of enzymes with broad substrate tolerance (59, 64). We have shown how the localized specificity of downstream carotenoid biosynthetic enzymes allowed pathway branches leading to whole sets of novel compounds to be opened when the substrate or product preferences of key upstream enzymes were altered. In a similar example demonstrating the localized specificity of other isoprenoid biosynthetic enzymes, a mutated hydroxylase located early in the monoterpene pathway of spearmint resulted in a plant with a completely shifted monoterpene

profile resembling that of peppermint (51). Other isoprenoid biosynthetic enzymes have very broad product specificity and synthesize multiple compounds (in one case, more than 50) from a single substrate (25, 46, 50, 60, 190, 229). Pathways harboring such enzymes are therefore highly branched and provide many possible avenues for further product diversification by directed evolution of downstream enzymes. Enzymes with broad specificity, a key feature of evolvable pathways, are found in the biosynthetic pathways of many other natural products, including alkaloids, polyketides, shikimate derivatives, nonribosomal peptides, and volatile esters (3, 26, 35, 169, 184, 188). It is likely that all these pathways will be readily diversified by laboratory evolution.

Laboratory evolution will also be useful in constructing hybrid pathways for new natural products. Nature parsimoniously achieves great increases in small-molecule product diversity through the biosynthesis of hybrid natural products. Mycophenolic acid, tocotrienols and tocopherols (vitamin E), cannabinoids, isoprenoid quinones, furanocoumarins, furanoquinolines, alizarin anthraquinones, and certain flavonoids and alkaloids are examples of natural products formed by the fusion of products from two or more distinct biosynthetic pathways (35, 127). Chemical synthesis of unnatural hybrids of natural products has given rise to molecules with potent biological activity, many of which possess combined properties of their individual natural product constituents (reviewed in references 132 and 208). The number of possible natural product hybrids is astronomical, and, like nonhybrid natural products, most have yet to be made. Accordingly, altering the specificity of enzymes catalyzing the fusion reactions that lead to hybrid products in nature may be a promising way to explore natural product diversity.

While laboratory evolution is likely in principle to be effective at exploring natural product diversity in a number of pathways, in practice, some experiments will be far easier than others. Thus, the above discussion is not complete without consideration of the technical issues involved in making and interrogating large libraries of mutant pathways. The first challenge is to create and express libraries of mutant enzymes in the context of a functional pathway. By virtue of their high transformation efficiency, fast growth, and the large number of available tools for genetic manipulation, *E. coli* cells are attractive for laboratory evolution experiments (and many other metabolic engineering experiments). Also, because *E. coli* does not synthesize carotenoids on its own, there is no background pool of carotenoids to contend with for laboratory evolution or combinatorial biosynthesis experiments with carotenoid biosynthetic pathways. *E. coli*, however, is not a universal protein expression machine, nor is it an ideal production host for all compounds. For example, it lacks the ability to glycosylate proteins. In addition, the precursors required for the biosynthesis of certain natural products, including many ployketides and isoprenoids, are present at low levels or not at all in *E. coli*. The use of engineered strains of *E. coli* with increased capacity for isoprenoid production (reviewed in reference 17) and the ability to synthesize complex polyketides (reviewed in reference 161) will overcome this limitation. Alternatively, libraries can be expressed and even constructed in other organisms such as *Saccharomyces cerevisiae*, for which a host of laboratory evolution tools exist (1, 2, 36, 37, 44, 45). Recently, an approach to the laboratory evolution of polyketide biosynthesis was demonstrated in which *E. coli* was used as a host for generating a plasmid library by in vivo recombination. This library was subsequently transformed into a special strain of *Streptomyces venezuelae* for expression and screening (99). This approach combines the ease of genetic manipulation of *E. coli* with the ability of *Streptomyces* to generate bioactive glycosylated polyketides. Many directed enzyme evolution experiments have used an analogous approach in which the mutant library was generated in *E. coli* but expressed in another, more suitable host such as *Bacillus subtilis* (43, 187, 240). This approach is then limited by the efficiency of transformation (often poor, especially for eukaryotic cells) and the ability to make cell lines that stably and reproducibly express the mutant sequences (and their pathway products).

Once the library of mutant pathways is constructed and expressed, it must be screened to identify those that produce a new or desired metabolite(s). As is the case for directed protein evolution, this part of the experiment is usually the most challenging and labor-intensive. In the examples discussed in this review, it was relatively easy to monitor the characteristic pigmentation of carotenoids (e.g. using simple, color-based visual screening of bacterial colonies on plates). It is possible that color-based screens will similarly prove useful for evolving flavonoid (195) or porphyrin (114) biosynthetic pathways, since these products are also pigmented. Nonetheless, most natural products do not generate fluorescent or UV-visible spectra that are characteristic and distinguishable in the presence of cell debris, and other strategies must be employed for screening. Screening becomes much more challenging when there are no characteristic spectral changes that can be measured in situ or when the desired products must be isolated and analyzed before they can be identified. (Even among visibly colored families of compounds, new structures are not identifiable by colony color screening unless their pigmentation differs markedly from that of the products of the native pathway. Additionally, cell color reflects the total product mixture, and evolved pathways must therefore generate a significantly different product spectrum from the native pathway in order to be apparent in a simple color-based screen.) Thus, more general methods for rapidly screening microbial clones for the production of a wide range of natural products are desirable. Ideally, the screens would require minimal purification or work-up of cells or lysates, would be highly sensitive and reproducible, and would be amenable to automation. Mass spectrometry (MS) can be used when a target product has a different mass or fragmentation pattern from the product(s) of the wild-type enzyme(s). Using MS, it is possible to screen up to 10,000 samples per day (182), and MS screening has been used to for the directed evolution of enzymes with altered product profiles or enantioselectivity (166, 168). However, due to the large capital investment required (>$1 million), automated, high-throughout MS-based screening equipment is found primarily in well-funded industrial research laboratories. Screening by high-performance liquid chromatography or thin-layer chromatography is also feasible but similarly requires sample pretreatment and is only high-throughout with parallel, automated instrumentation. Screening using nuclear magnetic resonance spectroscopy (NMR) has also been demonstrated (167). Up to 1400 samples per day could be analyzed by an

NMR spectrometer equipped with a commercially available flowthrough NMR probe head. Product concentrations, however, must be quite high for detection by NMR, and pretreatment is required. In some cases, pooling of samples increases the number of clones that can be screened, provided that there is sufficient sensitivity to detect the novel compounds (which are present at a lower concentration). Screens based on biological properties (e.g., antimicrobial activity or protein binding), widely used in drug discovery (24, 217, 221), could also be adapted to laboratory evolution studies.

CONCLUSIONS

In its short history, laboratory pathway evolution has produced more than 30 carotenoids that have never been seen in nature. With the ability to make new backbones in *E. coli* comes the potential to generate whole families of novel carotenoids through the action of wild-type or laboratory-evolved carotenoid-modifying enzymes on the new carbon scaffolds. Applied to other biosynthetic pathways, laboratory pathway evolution could allow researchers to access thousands of molecules that are difficult to produce in practical quantities by synthetic chemistry, are expensive to isolate from natural sources, or have not been found in nature.

Seeing is believing. Evolving carotenoid biosynthetic pathways in the laboratory has allowed us to witness first-hand their remarkable evolutionary potential. In the process, we have learned that carotenoid enzymes can acquire new specificities that allow them to accept different substrates, function in a foreign pathway, make a different product from the same substrate, and even give rise to new pathways. Furthermore, all of these changes can be effected in just one round of evolution and are usually brought about by a single amino acid substitution. As evolution proceeds, we will gain further, detailed information on how pathways diverge and, we hope, some elementary understanding of how the stringent specificity of enzymes and pathways we see in nature is achieved at the molecular level.

We wonder how far laboratory pathway evolution can be taken. For example, starting with the genes that make up an arbitrary biosynthetic pathway in nature, can we evolve any chemically possible pathway made up of the same basic transformations? We have seen that the carotenoid synthase CrtM can rapidly acquire new specificities, lose much of its original function, and yet quickly recover its original function via different mutational routes. This is an encouraging lesson: evolving an enzyme with narrowed specificity does not "paint it into a corner," evolutionarily speaking. It seems that one can abolish a particular specificity with ease but cannot abolish an enzyme's inherent ability to evolve. Thus, the enzymes that exist in 2005 may be perfectly good starting points for creating widely diverse pathways.

The dramatic functional changes we see in laboratory pathway evolution experiments remind us that natural product biosynthetic pathways are not merely a snapshot of history to be observed and documented. Rather, these pathways are continually changing entities whose evolutionary dynamics we are just beginning to probe. Laboratory evolution experiments with carotenoid pathways have given us an exciting glance at these dynamics. Future experiments promise both a greater understanding of how metabolic systems evolve and the discovery and optimization of biosynthetic routes to a host of molecules with diverse beneficial impacts on human health and well-being.

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