Chapter 14

Directed Evolution of Carotenoid Synthases for the Production of Unnatural Carotenoids

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Abstract

Directed evolution is a well-established strategy to confer novel catalytic functions to the enzymes. Thanks to the relative ease of establishing color screening, carotenogenic enzymes can be rapidly evolved in the laboratory for novel functions. The combinatorial usages of the evolvants result in the creation of diverse set of novel, sometimes unnatural carotenoids. This chapter describes the directed evolution of diapophytoene (C_{30} carotenoid) synthase CrtM to function in the foreign C_{40} pathway, and the use of the CrtM variants thus obtained for the production of novel backbone structures.

Key words: Directed evolution, Library construction, Error-prone PCR, Color screening, Combinatorial biosynthesis

1. Introduction

Industrial value of carotenoids ranges from anti-oxidants, natural pigments, hormonal drugs, and micronutrients. Functions of these carotenoids are based on their molecular structures (molecular size, solubility, effective number of conjugated double bonds, and presence/absence of functional groups or cyclic ends), justifying the effort to construct biosynthetic pathways for various carotenoids on demand. Dozens of carotenogenic enzymes have been functionally expressed in noncarotenogenic hosts, such as *Escherichia coli*, and the combinatorial expression of these enzymes often yields the pathways for novel carotenoids (1). When the desired functions are unavailable (or do not exist in nature), natural carotenoid enzymes can be engineered to fulfill them (2). Phytoene desaturases (3, 4), cyclases (3, 5), hydroxylases (6), and carotene synthases (7) have been successfully engineered to have altered functions. These "mutant" enzymes are assembled into the

José-Luis Barredo (ed.), *Microbial Carotenoids from Bacteria and Microalgae: Methods and Protocols*, Methods in Molecular Biology, vol. 892, DOI 10.1007/978-1-61779-879-5_14, © Springer Science+Business Media, LLC 2012

new pathways (natural or unnatural) to the targeted carotenoids. Carotenoid biosynthetic enzymes cannot be rationally engineered due to the lack of structural information. Instead, directed evolution can be well adapted for the creation of novel carotenoid biosynthetic enzymes (2, 8).

Directed evolution is divided in two experimental steps: library creation and functional screening (Fig. 1). Starting from one or a set of parental genes, a large set of genetic variants is prepared. Thus, created "enzyme library" is then subjected to the screening to isolate the clones with novel/desired catalytic functions. Due to the pigmentation of the pathway products, high-throughput color screening can be developed to isolate the desired enzymatic functions along the path. For the enzymes, such as desaturases, cyclases, and ketolases, one can often see the emergence of new functions. This is because these enzymes directly alter the photochemical properties of carotenoids and thereby change in functions can be visualized. For evolving enzymes catalyzing the invisible steps in carotenoid synthesis, several enzymes are co-expressed to visualize their cellular functions. As a case study, this chapter describes the directed evolution of diapophytoene (C_{30} carotenoid) synthase CrtM to function in the foreign C_{40} pathway. CrtM variants thus obtained are used for the production of novel backbone structures, which can be further diversified by the addition of decoration enzymes.

Before the directed evolution experiment, screening system needs to be set up: one should construct a carotenoid pathway, including the target enzymes as its component. The pathway should be designed so that the change in the target enzyme properties results in the change in colony hue. *E. coli* has been an exclusive host for evolving carotenogenic enzymes due to the lack of its background color, rapid growth, and wealth in the genetics. In the case of screening for phytoene synthase activity, geranylgeranyldiphosphate synthase (CrtE) is expressed to establish the precursor supply. Because the product of phytoene synthase is colorless, phytoene desaturase (CrtI) is additionally expressed to convert phytoene into red pigment, lycopene (see Note 1 and Fig. 2).

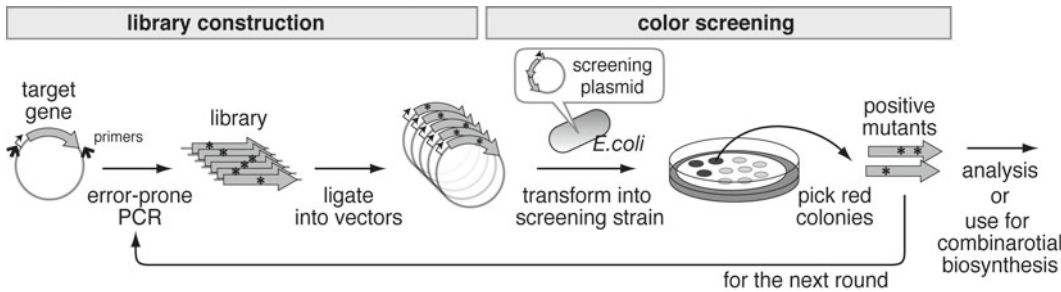

Fig. 1. General scheme of the directed evolution of carotenoid enzymes.

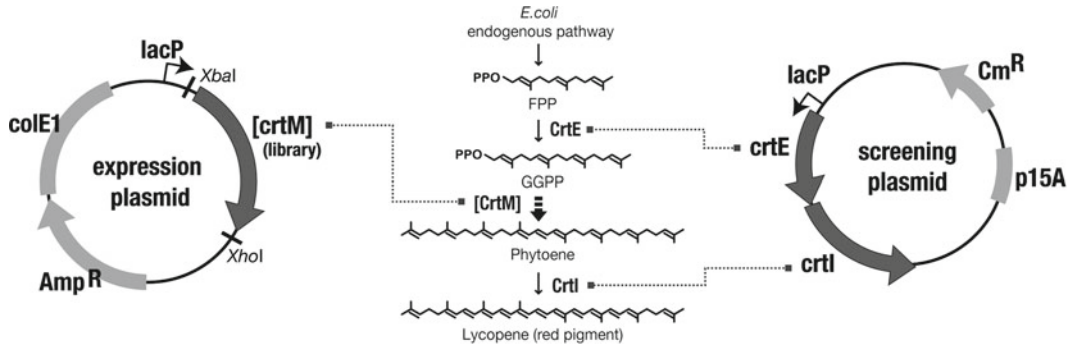

Fig. 2. Screening constructs for the CrtM mutants with phytoene synthase activity.

2. Materials

2.1. Library Creation (PCR Random Mutagenesis)

1. PCR primers forward and reverse (see Note 2).
2. pUC-crtM (see Note 3 and Fig. 2) (7).
3. *Taq* polymerase.
4. 10× *Taq* buffer.
5. 10× dNTP mixture: 2 mM each of dATP, dTTP, dCTP, dGTP. Prepare 50 µL aliquots of this mixture (to avoid excessive freeze/thaw cycles) and store at –20°C.
6. 10× $MnCl_2$ solution: 10 µM prepared in distilled water and stored at room temperature.
7. Nuclease-free water.
8. pUC-based vector (see Note 3) (7).
9. DNA clean and concentrator™—5 (Zymo Research Corporation, Orange, CA, USA).
10. Restriction enzymes *Xba*I and *Xho*I.
11. T4 DNA ligase.
12. TAE: 40 mM Tris–acetate, and 1 mM ethylene diamine tetraacetic acid (EDTA).
13. Ethidium bromide: 0.5 µg/mL in TAE.
14. Agarose gel: 0.7% LE agarose in TAE and ethidium bromide.
15. Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Orange, CA, USA).

2.2. Color Screening

1. Screening plasmid (for details, see Note 4 and Fig. 2).
2. Z-Competent *E. coli* Transformation Kit (Zymo Research Corporation, Orange, CA, USA) (see Note 5).
3. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) (see Note 5).

4. Bio Trace NT Membrane, 82-mm discs (PALL Gelman Laboratory, Ann Arbor, MI, USA).
5. Sterilized toothpicks.
6. Carbenicillin 1,000× stock: 50 mg/mL (see Note 6).
7. Chloramphenicol 1,000× stock: 30 mg/mL.
8. LB (Luria–Bertani): Add 10 g of bacto-tryptone, 5 g of yeast extract, and 5 g of NaCl into 1 L of deionized water. Autoclave at 121°C for 15 min (9).
9. LB-agar: LB and 20 g/L agar.
10. SOC: Add 20 g of bacto-tryptone, 5 g of yeast extract, and 0.5 g of NaCl into 990 mL of deionized water. Add 10 mL of a 250 mM solution of KCl. Autoclave at 121°C for 15 min. Just before use, add 5 mL of a sterile solution of 2 M $MgCl_2$ and 20 mL of a sterile 1 M solution of glucose (9).

3. Methods

3.1. Library Creation

There are a variety of established methods for the construction of an enzyme library (10). Among them, whole gene error-prone PCR is probably the most popular method. Addition of the Mn^{2+} into the PCR sample lowers the fidelity of the polymerase whereby inserting random base substitutions into the target sequence (11).

1. For each PCR sample, add to tube: 2 fmol template DNA (see Notes 7 and 8), 5 µL 10× *Taq* buffer, 5 µL 10× dNTP mixture, 5 µL of 5 µM each primers (to be 25 pmol in final concentration), 1 µL *Taq* polymerase (5 units), 5 µL 10× $MnCl_2$ solution (to be 10–50 µM in final concentration, see Note 9), and distilled water to a final volume of 50 µL.
2. Mix the sample by pipetting, and confirm all the solution is on the bottom.
3. Place the tubes in a thermal cycler, and run the PCR program below: (1) 5 min initial denature at 94°C. (2) 30 s denature at 94°C, 30 s annealing at the temperature designed for primers, 1 min extension at 72°C. Repeat for 25 cycles. (3) 10 min at 72°C for final extension.
4. Run the product for gel electrophoresis to estimate the yield of the full-length gene (see Notes 7–9).
5. Clean the PCR product by using a Zymo DNA clean and concentrator Kit. In parallel, clean up the vector in the same way.
6. Digest both PCR product and the expression vector with appropriate enzymes (*Xba*I and *Xho*I).

7. Gel-purify the digested samples using Zymoclean™ Gel DNA Recovery Kit (see Note 10).
8. Treat the mixture of the purified insert (100 ng) and vector (100 ng) with T4 DNA ligase for 2 h (400 units/reaction to total volume of 10 μL) (see Note 11).
9. Optionally, after the reaction, concentrate the ligation product by using a Zymo DNA clean and concentrator kit (see Note 12).

3.2. Color Screening

1. To transform the ligation product into *E. coli* XL1-Blue Z-competent cells harboring the screening plasmid (see Note 5), add 5 μL of the ligation product into 100 μL competent cell on ice.
2. Incubate on ice for 10 min.
3. Add 400 μL SOC medium and shake for 1 h at 37°C.
4. Meanwhile, prepare the screening plates: mount the nitrocellulose membrane on each of 5–10 LB agar plates containing chloramphenicol and carbenicillin. Handle the nitrocellulose membrane with tweezers, not with bare hands. The nitrocellulose membrane is used to provide a white background to facilitate visualization of colony color.
5. Spread all the transformants on the screening plates (see Notes 13 and 14).
6. Incubate the plates at 37°C until the colonies become visible in size (see Note 15).
7. Leave the plates at room temperature for additional 12–36 h until the color develops (see Note 16).
8. When the color of colonies became distinguishable, determine which ones should be the positive variants (see Notes 17–19).
9. Pick all of the pink (positive) clones using sterile toothpicks and inoculate into a 2 mL LB liquid media containing chloramphenicol and carbenicillin. Shake the culture overnight at 37°C.
10. Miniprep the culture to collect the mutant plasmids.
11. At this point, plasmid contains both the mutant plasmid and the screening plasmid. To remove the latter, retransform the plasmid into *E. coli* strain (cloning strains without plasmids). Spread it on an LB agar plate containing carbenicillin. Pick the colony with no pigmentation, culture in LB liquid media, and miniprep to obtain the pure mutant plasmid.
12. For routine analysis of the mutants, determine the genotype of the mutant genes by DNA sequencing. Cellular function of the variants can be analyzed by the co-expression of various carotenogenic genes, followed by the pigment extraction and product analysis using HPLC-MS.

3.3. Toward the Novel Carotenoids

Sequence analysis of the above-obtained CrtM mutants revealed two amino acid residues (F26, W38) that control the size specificity of this enzyme (12, 13). In a different directed evolution experiment, farnesyl diphosphate synthase (FDS) from *Bacillus stearothermophilus* was also engineered into geranylfarnesyl diphosphate (C_{25}PP) synthase (14). Co-expression of this FDS mutant with size mutants of CrtMs resulted in the production of the novel phytoene-type carotenoid with C_{45} ($C_{25}+C_{20}$) and C_{50} ($C_{25}+C_{25}$) backbones (7). Further addition of carotenoid desaturases CrtI resulted in the production of diverse set of C_{45} and/or C_{50} carotenoids without protein engineering (15). Many of the downstream enzymes were shown to accept various different carotenoids. This way, structural variation of the carotenoids can be easily expanded by the addition of laboratory-evolved carotenogenic enzymes in a toolbox of the combinatorial biosynthesis.

4. Notes

1. For the convenience in mutant isolation and subsequent analysis, we prefer to place the target gene (the random library of *crtM*) on a pUC-based high copy expression vector (Fig. 2). The additional genes (*crtE* and *crtI*) are grouped into another pACYC-based vector. By physically separating the library (in expression vector) from other genes in the pathway (grouped in screening plasmid), one can significantly reduce the false positive clones by the accidental incorporation of mutations in nontarget genes during DNA handing. Note that this two-plasmid system has some drawbacks: first, one must prepare the competent cells harboring the screening plasmid by his/her own (for this purpose, we recommend Z-Competent *E. coli* Transformation Kit; see Note 5). Secondly, every time after the color screening, the screening plasmids must be removed from the miniprep of the positive colonies (see Subheading 3.2, step 11).
2. PCR primers are designed to share the same or similar melting temperatures. They are used at 5 μM and stored at −20°C. The primers for error-prone PCR are designed to anneal to the outside of the reading frame of the gene so that the entire reading frame are randomized. In this case, however, regulator sequence (promoter and ribosome binding site) is also under mutagenesis. In the situation where the mutations in the regulation sequence could lead to the false-positives, design the forward/reverse primer to anneal to the N/C terminus of the reading

frame, respectively. In this case, the priming sites of the target genes are virtually "masked" from the mutagenesis.

3. *crtM* gene is placed under *lac* promoter (see Note 20) followed by optimized ribosome binding site (AGGAGG). *crtM* library is to be ligated into the *Xba*I–*Xho*I site of the plasmid.
4. Two genes (*crtE*, *crtI*) are placed under *lac* promoter (see Note 20) followed by optimized ribosome binding site (AGGAGG).
5. Z-competent cells harboring screening plasmids. Z-Competent *E. coli* Transformation Kit is highly recommended to make competent cells of your own. Following the simple protocol, the transformation efficiency reliably exceeds 10^8 transformants/μg plasmid. With this efficiency, $>10^4$ colonies (variants) can be screened for single transformation using 1/5 of the ligation product.
6. The use of carbenicillin instead of ampicillin is important to prevent the formation of satellite colonies. Screening sometimes requires 2–3 days to fully develop the colony colors, and ampicillin does not stay effective that long.
7. In the given (fixed) concentration of Mn^{2+}, the average mutation rate of the library is proportional to the effective cycle numbers of the PCR. The most convenient way to control the cycle number is to change the amount of target DNA in the PCR. In theory, 10-fold decrease in template in error-prone PCR result in $\log_2 10$ ~ca. 3.3-fold elevation in mutation rate, given that final yield of PCR stay the same.
8. With a high amplification yield of error-prone PCR (>1,000-fold), one can keep the library free from contaminating the unamplified wild-type sequence.
9. Mutation rates of the library can be controlled by the concentration of Mn^{2+} in a PCR reaction. Typical concentration ranges for Mn^{2+} is from 0 to 100 μM. Higher concentration results in the higher mutation rate of the resultant library. In the high side (>200 μM), polymerase reaction is inhibited, resulting in the low amount or no product.
10. This step is important to remove the template plasmid. In addition to this size-fractionation, one can further eliminate the template plasmid by the treatment of PCR product with *Dpn*I, which selectively digests plasmid-borne methylated DNA, in prior to the gel purification.
11. Addition of 10 mM ATP in the reaction mixture would increase the ligation efficiency.
12. After purification, ligation can be stored at –20°C without losing the transforming efficiency (library size). In case where

the larger library is necessary, transform the (concentrated) ligation into commercial super-competent cells and propagate the transformant in a single mixed culture for 12 h. Miniprep this mixed culture to prepare plasmid library ready for transforming to screening strains.

13. Unlike agar surface, nitrocellulose membrane is too smooth to catch the cell well by using spreader. This way, most of the transformant are wiped out to the edge of the plate, forming the colonies only on the perimeter area of the membrane. Instead, directly spill the diluted transformant culture (1 mL/plate) on the screening plate. Make sure the media covers the entire surface of the plate. Wait until the surface gets dried and then put them into the incubator (37°C).
14. For the better color development, limit the colony density to be less than 500 colonies/plate by adjusting the culture to be spilled on each plate.
15. The time needed for colony formation depends both on the type of *E. coli* strain and the type of plasmids to be transformed. XL1-Blue cells harboring two plasmids in Fig. 2 take 20–24 h to form a colony at 37°C.
16. Two-step incubation is necessary because pigment production (color development) of C_{40} carotenoids is very low at 37°C. On the contrary, incubation/shaking at 37°C is desirable for growth and plasmid collection.
17. Count and record the number of the colonies formed on the plate. The total number of the colonies is referred to as "screening size."
18. In properly designed screening, clones of interest can be easily identified by naked eye. For the quantitative ranking and analysis of the performance of each of the entire library, consider the use of digital image analysis (16).
19. You might find nothing that appears positive. If that is the case, it could indicate (1) quality or size of the library was not high enough (failure in library creation), (2) screening was not good enough to visualize the activity (failure in screening design), or simply, (3) the enzyme was not capable of acquiring the activity by a couple of amino acid substitution (goal too ambitious).
20. In this system, no IPTG-induction is necessary; the leaky expression from *lac* promoter stably supports the expression needed for cellular pigmentation. Too much expression of carotenoid genes is sometimes toxic or even lethal. For instance, CrtE slows the cell growth, while CrtI is almost lethal upon full induction.

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