Alteration of Product Specificity of *Rhodobacter sphaeroides* Phytoene Desaturase by Directed Evolution*

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Phytoene desaturases occurring in nature convert phytoene to either neurosporene or lycopene in most eubacteria. Approximately 10% of known phytoene desaturases, as in *Rhodobacter*, produce neurosporene, whereas the rest produce lycopene. These two types of enzymes, although similar in function, have relatively low similarity (below 60%) in terms of nucleotide or amino acid sequence. The mechanism controlling the product specificity of these enzymes is unclear. Here we used directed evolution to change the product of *Rhodobacter sphaeroides* phytoene desaturase (*crtI* gene product), a neurosporene-producing enzyme, to lycopene. Two generations of random mutagenesis were performed, from which three positive mutants were isolated and sequenced. We then used site-directed mutagenesis to determine the effect of each amino acid change. Gathering information from random mutagenesis, we further recombined the beneficial mutations by site-directed mutagenesis and increased the percent of lycopene production to 90%.

Directed evolution has been used in many aspects of biological systems, such as protein engineering (1), vaccine development (2), biochemical production (3), and metabolic engineering (4). In general, it involves fast mutation of the target gene(s), followed by an efficient screening or selection system for a desirable trait. Products with improved functions or novel compounds were generated using this approach (5). Directed evolution can also be used to generate desirable mutants from which sequence alternation can be correlated with function. In this study, we demonstrate an example of directed evolution for the alteration of product specificity. Analysis of point mutations sheds light on the mechanism determining the product specificity.

Phytoene desaturase in eubacteria (*crtI* gene product) (6) is an important enzyme in the carotenoid pathway. It catalyzes the desaturation of phytoene to either neurosporene or lycopene (see Fig. 1). Lycopene is a red pigment and an antioxidant that has been shown to have preventive effects against certain cancers (7). The yellow carotenoid neurosporene is structurally different from lycopene by one double bond with one-step earlier termination in the desaturation reaction (see Fig. 1). These compounds are metabolites downstream from geranylgeranyl diphosphate (GGPP)\(^1\) in the isoprenoid pathway via phytoene synthase encoded by *crtB*. About 10% of analyzed phytoene desaturases produce neurosporene, such as the enzymes from *Rhodobacter*, whereas the rest produce lycopene, such as in *Agrobacterium aurantiacum* (8), *Erwinia* sp. (9), and other photosynthetic bacteria (10). At the DNA level, the *crtI* gene encoding phytoene desaturase of the *Rhodobacter* species is significantly different from the ones from other organisms. Between the *crtI* genes of *Rhodobacter* and others, the amino acid sequence similarity is below 60% although there are some conserved regions, especially in the C-terminal domain (11). Because it is highly distinct between these two classes of phytoene desaturases, it would be relatively difficult to perform DNA family shuffling (12) or other in vitro recombination methods.

Because these two types of phytoene desaturases share relatively low nucleotide identity or amino acid sequence similarity (below 60%), it cannot be deduced from a sequence comparison which are the residues that determine product specificity. Recently, Schmidt-Dannert et al. (5) mutated *Erwinia* phytoene desaturase to produce a more desaturated compound, 3,4,3',4'-tetrahydroxylycopene (see Fig. 1). To determine the amino acid residue(s) important for controlling product specificity in phytoene desaturases, we used error-prone PCR mutagenesis to evolve *Rhodobacter sphaeroides* phytoene desaturase to produce lycopene. The mutated amino acids were individually tested by site-directed mutagenesis for their importance in determining product specificity.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Materials**—The *crtI* gene encoding phytoene desaturase was cloned from *R. sphaeroides* ATCC17025, according to the published *crtI* sequence of another *R. sphaeroides* NCIB 8253 (13).

The 1.7-kb *crtI* fragment was amplified from the genomic DNA using PCR with a pair of designed degenerate primers (5'-TGGGGTACCTG-CATGTCATTCCTC-3' and 5'-TGCTCTAGATCATTCCGCGGCAAG-3'). The PCR product was ligated into the pCR2.1 vector (Invitrogen Inc., Carlsbad, CA) by the TA-cloning technique and cotransformed into competent TOP10 host (Invitrogen, Inc.) with plasmid pCW18, which overexpresses the necessary enzymes for producing the substrate, phytoene, in *Escherichia coli* (Fig. 1). The plasmid pCW18 is spectinomycin-resistant and harbors the *dxs* gene encoding 1-deoxy-d-xylulose 5-phosphatase synthase from *E. coli*, *gps* encoding GGPP synthase from *Archaeoglobus fulgidus* (14), and *crtB* encoding phytoene synthase from *Erwinia uredovora* on the vector pCL1920 (15). This plasmid was constructed by destroying the *crtI* gene on pCW9 (containing *dxs*, *gps*, and *crtBl* from *E. uredovora*) (16) with BarnHI and then filled in the overhang and self-ligated. Among the yellow transformatants due to the activity of the *R. sphaeroides* *crtI* gene, one was picked, and the plasmid harboring *crtI* was isolated, sequenced, and designated as pCW19. The sequence of our cloned gene is 87% identical to the published one for *R. sphaeroides* NCIB 8253 (13) at the nucleotide level and around 93% similar at the amino acid level. The plasmid pCW19 was found to contain the *crtI-lacZa* fusion with 18 amino acids (32 nucleotides) of *crtI* truncated at the C terminus (see Fig. 3A). However, the yellow pigment was confirmed to be neurosporene by high performance liquid chromatography (HPLC) measurement.

**Error-prone PCR Mutagenesis**—Random mutagenesis of the *R. sphaeroides* *crtI* gene on the plasmid pCW19 was performed using methods...
Directed Evolution of Phytoene Desaturase

RESULTS

Directed Evolution of Phytoene Desaturase by Random PCR Mutagenesis—Similar to the two-plasmid system in our previous work (4), we cloned the target \( \text{crtI} \) gene from \( R. \text{sphaeroides} \) ATCC17025 on one plasmid and the rest of the pathway genes (\( \text{dxx}, \text{gps}, \) and \( \text{crtB} \)) onto another compatible plasmid (pCW18). Random mutations were introduced to the \( \text{crtI} \) gene by PCR under mutagenic conditions. The 1.7-kb PCR fragment contains the upstream promoter region, the open reading frame of the \( \text{crtI} \) gene, and the downstream region. After mutation, restriction digestion, purification, and re-ligation, the plasmid DNA was transformed into the host strain TOP10 containing pCW18. The colonies were grown on plates to screen for different colors due to the accumulation of different carotenoids. Approximately 7,500 colonies were screened on the plates visually for the first generation of directed evolution. The \( E. \text{coli} \) cells expressing the wild-type \( R. \text{sphaeroides} \) phytoene desaturase appeared deep yellow. One colony showed a color change from yellow to slightly golden. This candidate mutant was grown in LB medium with the appropriate antibiotics, and the products were identified by HPLC. Results showed that this mutant strain produced a mixture of carotenoids, including neurosporene (45%), lycopene (32%), and some amounts of \( \zeta \)-carotene (3% and 6%) (Fig. 2A). The plasmid harboring the mutated \( \text{crtI} \) gene was isolated and designated as pCW19 m1.

We proceeded to perform the next mutagenesis by error-prone PCR using pCW19 m1 as a template. About 10,000 colonies were screened, and their colors were compared with the control harboring pCW19 m1. Twenty colonies showed a color change, and two of them appeared completely pink. These two mutants were grown in LB medium with antibiotics, and the carotenoids produced were subjected to HPLC analysis. Both mutants produced a mixture of carotenoids with the majority being lycopene (77 and 78%) and small amounts of neurosporene (20 and 16%) and \( \zeta \)-carotene (3 and 6%) (Fig. 2A). The two plasmids harboring the mutated \( \text{crtI} \) genes from the second mutant generation were isolated and designated as pCW19 m2 and pCW19 m3, respectively.

Sequence Analysis—The three mutants m1, m2, and m3 were sequenced. Three point mutations were found on m1. Two of them led to the amino acid changes, F220S and E508G, whereas the third one caused the early truncation of the protein by changing the amino acid arginine into a stop codon (see Fig. 3A and Table I). Three pairs of mutagenic primers for site-directed mutagenesis were designed to investigate the effect of each point mutation. We found that the change to stop codon caused the most significant change to product distribution (Fig. 2B), followed by E508G, and the mutation F220S had no effect on product distribution. These results are summarized in Table I and shown schematically in Fig. 3B.

Four additional point mutations were found in mutant m2 compared with the parental template (m1), which lead to four amino acid changes, H12Q, V68D, F166I, and M402T. Again, site-directed mutagenesis was performed to determine the effect of each point mutation. We found that V68D and F166I were the two beneficial mutations that help the conversion of product from neurosporene to lycopene (see Fig. 2C and Fig. 3B). The mutation H12Q has little effect, whereas M402T has a negative effect on percent lycopene production.

Mutant m3 has three additional point mutations compared with the m1 template, including one amino acid change,
L148H, and two silent mutations that cause no amino acid changes. We verified the effect of L148H by introducing this mutation into m1 by site-directed mutagenesis and, as expected, found that it increased the production of lycopene dramatically to about 80%. Moreover, we also introduced this mutation alone into the wild-type template pCW19, and the effect is also prominent (52% lycopene) (Fig. 2D).

**Protein Evolution by Site-directed Mutagenesis**—Based on the information obtained from two generations of directed evolution by random mutagenesis, we know that several amino acid changes were beneficial for achieving higher production of lycopene. The two final mutants generated from random mutagenesis, m2 and m3, showed nearly no difference in color when compared with the positive control harboring pCW9 that overexpressed the lycopene-producing CrtI from *E. uredovora* (16), although they still synthesize small amounts of neurosporene. Thus, this visual limitation hinders the further use of color screening to isolate the next-generation mutants by evolution.

However, we can resort to site-directed mutagenesis based on information generated from directed evolution. Our results showed that L148H had the most significant positive effect among all the mutations, followed by F166I, R514Stop, V68D, and E508G. On the other hand, M402T had a negative effect for lycopene production whereas F220S and H12Q have negligible effects. To optimize lycopene production, we then combined all the positive mutations and eliminated negative mutations by site-directed mutagenesis. First, L148H was introduced into the mutant m2, because it does not have this mutation, and its lycopene production is already high (80%). The result showed that L148H further increased the lycopene production up to 90% (see Fig. 2D and Fig. 3B).

We suspected that we could further increase the production by removing the negative mutation M402T. To do so, we started from the mutant m1 and introduced mutations V68D and F116I and found the lycopene production of this strain was just slightly higher than that of m2 or m3. Finally, we incorporated L148H into the above strain, and its lycopene production level was around 90% (Fig. 3B). Note that this level of lycopene production was achieved with or without the negative mutation M402T. It appears that the negative effect was compensated by multiple positive mutations.

**DISCUSSION**

In this work, we evolved the *R. sphaeroides* phytoene desaturase to change its product from neurosporene to lycopene. We used site-directed mutagenesis to investigate the relative importance of each mutation to product specificity and combined the positive mutations. By combining error-prone PCR and site-directed mutagenesis, we changed the neurosporene-producing enzyme to lycopene-producing. We conclude that there are two important regions of this enzyme in determining the product distribution from neurosporene to lycopene. One is in the C-terminal region; the other is close to the N terminus (Val<sup>68</sup>, Phe<sup>166</sup>, and Leu<sup>148</sup>) that may contain the substrate-binding motifs.

Although many carotenoid biosynthetic genes have been cloned from various organisms, no crystal structures of these enzymes, including phytoene desaturase, are available so far. Previously, the hydrophobic C-terminal domain of the phytoene desaturase was identified to be conserved among carotenoid desaturases and required for the interaction in the dehydrogenation reaction (11). Schmidt-Dannert et al. (5) also showed that one point mutation in this domain changes product distribution. Sequence analysis of our mutant m1 showed two positive mutations near this C-terminal hydrophobic domain. The major one introduced a stop codon; the other caused the amino acid change E508G. The *crtI* genes of *Rhodobacter* sp. are longer in the C terminus compared with those of the organisms that produce lycopene. Thus, the C-terminal domain appears to be important in stopping the desaturation process at the level of neurosporene. Without this domain, the desaturation process continues to lycopene.

From the number of the colonies in the screening process, we noticed that it is relatively difficult to obtain a positive mutant in the first generation of random mutagenesis. It may be because of mutating a larger fragment (1.7-kb) by error-prone PCR, the use of the color screening system, or the rigidity of the enzyme. Once a mutant was obtained, several positive mutants...
List of the R. sphaeroides phytoene desaturase mutants generated by random and site-directed mutagenesis

| Gene  | Parental template | DNA mutation | Amino acid mutation | Lycopene production (%) |
|-------|-------------------|--------------|---------------------|-------------------------|
| m1    | WT                | T659C        | F220S               | 39                      |
|       |                   | A1523G       | E508G               |                         |
|       |                   | A1540T       | R514stop(opal)      |                         |
| G1.1  | WT                | T659C        | F220S               | 0                       |
| G1.2  | WT                | A1523G       | E508G               | 10                      |
| G1.3  | WT                | A1540T       | R514stop(opal)      | 21                      |
| m2    | m1                | T36A         | H12Q                | 80                      |
|       |                   | T920A        | V68D                |                         |
|       |                   | T4986A       | F166I               |                         |
|       |                   | T1205C       | M402T               |                         |
| G2.1  | m1                | T36A         | H12Q                | 37                      |
| G2.2  | m1                | T203A        | V68D                | 53                      |
|       |                   | T496A        | F166I               | 65                      |
| G2.4  | m1                | T1205C       | M402T               | 23                      |
| G3.1  | G2.2              | T4986A       | F166I               | 82                      |
| m3    | m1                | T443A        | L148H               | 82                      |
|       |                   | T1086A       | L362L               |                         |
|       |                   | G1524A       | G508G               |                         |
| G2.5  | m1                | T443A        | L148H               | 79                      |
| G2.6  | m1                | T1086A       | L362L               | 37                      |
| G2.7  | m1                | G1524A       | G508G               | 37                      |
| G1.4  | WT                | T443A        | L148H               | 53                      |
| m2    | m2                | T443A        | L148H               | 90                      |
| G4.1  | G3.1              | T443A        | L148H               | 89                      |

Rhodobacter sp., have Val, Leu, or Met. We also noticed that the second beneficial mutation on m2, F166I, falls into one of the flavin adenine dinucleotide-binding sequence motifs (Fig. 3A), which belong to the class of glutathione reductase (20). However, the other two beneficial mutations, V68D and L148H, are in the regions that are not identified previously to be associated with any properties. We suspect that these two amino acid residues may be in the domains that are involved in the substrate binding. Further investigation of these mutations would elucidate the mechanisms that determine product specificity.

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