

# Molecular breeding of carotenoid biosynthetic pathways

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The burgeoning demand for complex, biologically active molecules for medicine, materials science, consumer products, and agrochemicals is driving efforts to engineer new biosynthetic pathways into microorganisms and plants. We have applied principles of breeding, including mixing genes and modifying catalytic functions by in vitro evolution, to create new metabolic pathways for biosynthesis of natural products in *Escherichia coli*. We expressed shuffled phytoene desaturases in the context of a carotenoid biosynthetic pathway assembled from different bacterial species and screened the resulting library for novel carotenoids. One desaturase chimera efficiently introduced six rather than four double bonds into phytoene, to favor production of the fully conjugated carotenoid, 3,4,3',4'-tetrahydrolycopene. This new pathway was extended with a second library of shuffled lycopene cyclases to produce a variety of colored products. One of the new pathways generates the cyclic carotenoid torulene, for the first time, in *E. coli*. This combined approach of rational pathway assembly and molecular breeding may allow the discovery and production, in simple laboratory organisms, of new compounds that are essentially inaccessible from natural sources or by synthetic chemistry.

Keywords: carotenoid, molecular breeding, metabolic engineering, in vitro evolution

Natural products comprise an enormous diversity of chemical structures and biological functions. Unfortunately, many potentially useful compounds are found in only trace quantities in their natural sources and are difficult or impossible to synthesize chemically. And however rich this pool of natural structures, it is but a tiny fraction of the structures that could be made biologically. This essentially infinite bank of possibly functional molecules is a compelling target for biological design. Driving the field of metabolic engineering is the hope that recombinant cells can serve as biosynthetic factories as well as sources of new molecular diversity<sup>1-4</sup>. Biosynthetic enzymes and catalytic modules can be recombined into new pathways for the synthesis of natural and novel metabolites<sup>3,4</sup>. However, reliance on finding the appropriate catalytic functions in nature needlessly limits the compounds that can be synthesized in engineered organisms.

The principles of breeding and in vitro evolution can be used to access natural product diversity rapidly and in simple laboratory organisms such as *Escherichia coli*. To "breed" new biosynthetic pathways we can mix and match genes from different sources, even from unrelated metabolic routes, and at the same time create new biosynthetic functions by random mutagenesis, recombination, and selection<sup>5-8</sup>, all in the absence of detailed information on enzyme structure or catalytic mechanism. Because the gene functions introduced into the recombinant organism are not coupled to its survival, this approach can freely explore the space of possible product compounds, both natural and otherwise.

Carotenoid biosynthesis provides an excellent system with which to demonstrate this concept of molecular pathway breeding. Carotenoids make up a diverse class of natural pigments that are of interest for pharmaceuticals, food colorants, and animal feed and nutrient supplements. More than 600 different carotenoids are produced by microorganisms and plants. The discovery that these natural products can play a role in the prevention of cancer and chronic disease (mainly because of their antioxidant properties) and, more

recently, that they exhibit significant tumor suppression activity as a result of specific interactions with cancer cells has boosted interest in their pharmaceutical potential<sup>9-12</sup>. However, only a few can be obtained in useful quantities by chemical synthesis, extraction from their natural sources, or microbial fermentation<sup>13</sup>.

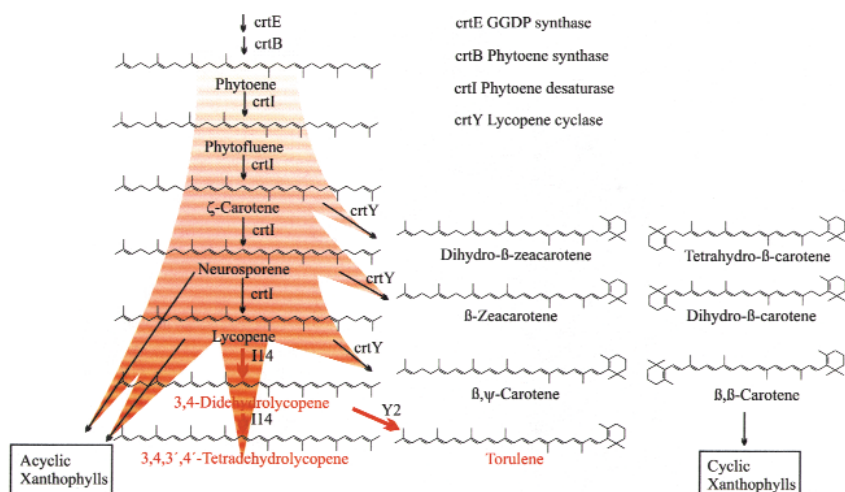
A number of carotenogenic genes have been cloned from microorganisms and plants and expressed in *E. coli*, thereby allowing the recombinant biosynthesis of different acyclic and cyclic carotenoids and oxo-carotenoids<sup>14,15</sup>. Various carotenoids can be produced in recombinant microorganisms by combining biosynthetic genes from different organisms<sup>16</sup>. A much wider range of carotenoids can be produced, however, by breeding biosynthetic genes and evolving new enzyme functions.

Carotenoid biosynthesis in a noncarotenogenic microorganism such as *E. coli* requires extension of the general terpenoid pathway with the genes for geranylgeranyl diphosphate (GGDP) synthase (*crtB*) and phytoene synthase (*crtE*) for the production of the C<sub>40</sub> carotenoid phytoene (Fig. 1). Subsequent desaturation by phytoene desaturase (*crtI*) and further modifications catalyzed by, e.g., cyclases, hydroxylases, and ketolases, result in the production of different carotenoids<sup>17</sup>. To date, most of the carotenogenic genes employed in recombinant biosynthesis are derived from either *Rhodobacter* or *Erwinia* species<sup>18,19</sup>.

To enable biosynthesis of new carotenoids in *E. coli* we targeted phytoene desaturase (*crtI*) and lycopene cyclase (*crtY*) for in vitro evolution. These enzymes are located at important branchpoints of the carotenoid biosynthetic pathway and determine the types of acyclic or cyclic carotenoids produced (Fig. 1).

## Results and discussion

**Molecular breeding of acyclic carotenoid biosynthesis.** Phytoene desaturases either introduce two desaturations in phytoene to produce  $\zeta$ -carotene, as in plants and cyanobacteria, three desaturations to produce



**Figure 1.** C<sub>40</sub> carotenoid biosynthesis branches into a variety of pathways to acyclic and cyclic carotenoids for which biosynthetic genes from bacteria have been cloned (for a review see refs 15, 17). Red arrows indicate how the central desaturation pathway has been extended to obtain the fully conjugated 3,4,3',4'-tetrahydrolycopene and subsequent branching of this pathway for the synthesis of torulene.

neurosporene, as in *Rhodobacter*, or four desaturations to produce lycopene, as in *Erwinia* and other photosynthetic bacteria<sup>20</sup>. The desaturase from *Neurospora crassa* introduces five double bonds into phytoene to synthesize 3,4-didehydrolycopene<sup>21</sup>. A desaturase capable of introducing six double bonds into phytoene would lead to the production of the fully conjugated carotenoid 3,4,3',4'-tetrahydrolycopene. The phytoene desaturase from *Erwinia uredovora* has been shown to synthesize small amounts of 3,4,3',4'-tetrahydrolycopene under certain conditions<sup>22,23</sup>. Our first goal was to extend the desaturation pathway by evolving an efficient six-step desaturase in order to generate 3,4,3',4'-tetrahydrolycopene as the major carotenoid in *E. coli* (Fig. 1).

To create variant enzyme libraries in the context of a biosynthetic pathway in *E. coli* requires co-transformation with two plasmids that together are stably propagated. Genes that produce the carotenoid precursors that serve as substrates for the target enzyme were cloned on a pACYC184-derived plasmid. Genes for the enzymes that were subjected to evolution in vitro were cloned on a pUC19-derived plasmid. All enzymes were individually expressed under the control of a *lac* promoter followed by an optimized Shine–Dalgarno sequence.

*Escherichia coli* cells co-transformed with pAC-*crtE*<sub>EU</sub>-*crtB*<sub>EU</sub>, expressing the GGDP synthase (*crtB*<sub>EU</sub>) and the phytoene synthase (*crtE*<sub>EU</sub>) from *E. uredovora* (EU), and with pUC-*crtI*<sub>EU</sub> or pUC-*crtI*<sub>EH</sub> expressing the phytoene desaturases (*crtI*) from *E. uredovora* and *E. herbicola* (EH), respectively, produced lycopene as the exclusive carotenoid (Fig. 2A). These cells appeared orange to orange-red on plates and in liquid culture. A library of desaturases generated by in vitro homologous recombination (DNA shuffling<sup>5</sup>) of the genes from *E. herbicola* and *E. uredovora* was transformed into phytoene-synthesizing *E. coli* JM101 harboring pAC-*crtE*<sub>EU</sub>-*crtB*<sub>EU</sub>.

Colonies were transferred to nitrocellulose membranes, which provide a white background for visual screening of the clones based on color. Approximately 10,000 colonies were screened; 30% appeared white as a result of inactivation of the desaturase. Twenty colonies were yellow, indicating the presence of carotenoids with fewer conjugated double bonds than lycopene. In addition, we identified one pink clone (I14), suggesting the introduction of additional double bonds into lycopene by this mutant. Analysis of cell extracts by high-pressure liquid chromatography (HPLC) showed that the desaturase of I14 introduces two double bonds in lycopene, which leads to the accumulation of the terminal desaturation product 3,4,3',4'-tetrahydrolycopene in addition to lycopene (Fig. 2B). Yellow mutant I25, in contrast, introduces two double bonds in phytoene (Fig. 2C). Reflecting the stepwise

nature of desaturation, I25 synthesizes neurosporene and lycopene in addition to the main product, ζ-carotene (whereas wild type produces only lycopene—Fig. 2A). Although it is reported that wild-type *Erwinia* desaturases can produce dehydrolycopenes<sup>22,23</sup>, we do not observe even trace amounts under these cultivation conditions.

Sequence analysis of the I25 desaturase showed two amino acid changes, R332H and G470S, in the sequence of the *crtI*<sub>EU</sub>, and no recombination. G470S is located in a hydrophobic C-terminal domain that is thought to be involved in substrate binding and the dehydrogenation reaction, and is conserved among carotenoid desaturases<sup>24</sup>. In mutant I14, the N terminus (residues 1–39) of the desaturase from *E. uredovora* is replaced with that of *E. herbicola*, which differs in only four residues (P3K, T5V, V27T, L28V). The I14 desaturase also contains two amino acid substitutions, F231L and A269V. We constructed two chimeras to determine whether the N-terminal recombination or the point mutations (or both) were responsible for the altered catalytic activity of mutant I14. Chimera I contained

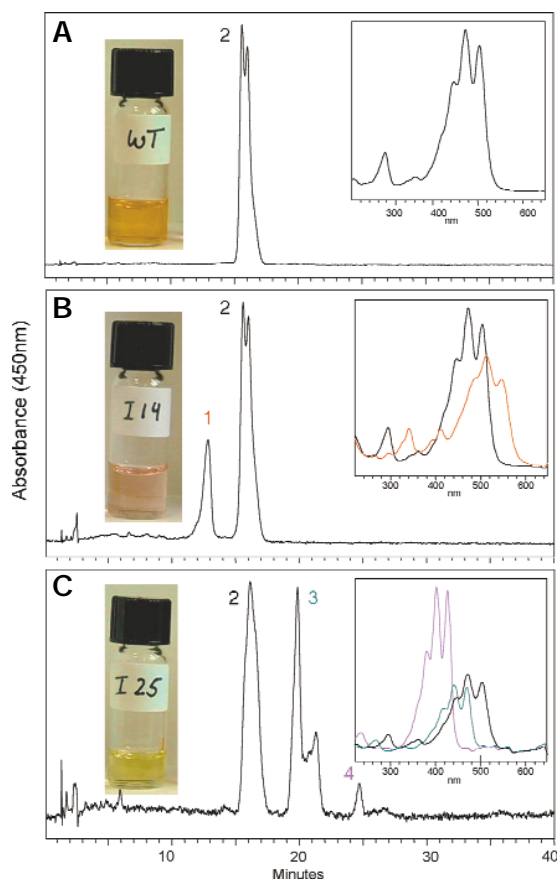
only the recombinant N terminus, and chimera II contained only the two amino acid changes. Surprisingly, only chimera I exhibited the altered catalytic activity of mutant I14. The N terminus comprises a typical dinucleotide-binding domain (GXG(X)<sub>2</sub>A/G(X)<sub>3</sub>A(X)<sub>6</sub>G) (ref. 25) not previously associated with substrate specificity. Thus, cofactor binding (FAD in *Erwinia* desaturases<sup>22</sup>) appears to play an important role in controlling substrate specificity.

**Molecular breeding of cyclic carotenoid biosynthesis.** Starting from neurosporene in *Rhodobacter* or lycopene in other photosynthetic bacteria, diverse acyclic carotenoids are synthesized by further desaturation, hydroxylation, and methylation. Yet other bacteria, e.g. *Erwinia*, synthesize cyclic carotenoids from lycopene. These modifying enzymes show a high degree of promiscuity that allows them to act equally well on neurosporene and lycopene in engineered pathways<sup>26–29</sup>. We reasoned that carotenoids with a further extended chromophore would also be modified by these enzymes or their evolved variants, leading to novel carotenoids in *E. coli*. The next step, therefore, was to generate new pathways for the biosynthesis of cyclic carotenoids by in vitro evolution of the cyclase.

Bacterial lycopene cyclases usually introduce β-ionone rings at both ends of lycopene to produce β,β-carotene<sup>30</sup> (Fig. 1). However, when neurosporene is produced by a three-step desaturase from *Rhodobacter*, or ζ-carotene is produced by a two-step desaturase from *Synechococcus* sp. in an engineered pathway, the cyclase is capable of cyclizing not only the ψ-end group (as in lycopene and at one end of neurosporene) to the β-end group, but also the 7,8-dihydro-end group (as at one end of neurosporene and in ζ-carotene) to the 7,8-dihydro-end group<sup>28</sup> (see Fig. 1 for carotenoid structures). Synthesis of the respective monocyclic intermediates demonstrated that the enzyme acts on the two ends separately. The proposed reaction mechanism for cyclization involves only the double bonds C1–C2 (C1'–C2') and C5–C6 (C5'–C6'), which agrees with the observed broad substrate specificity<sup>31</sup>. Hence, we reasoned that breeding could generate a lycopene cyclase that efficiently cyclizes 3,4-didehydrolycopene, the precursor of 3,4,3',4'-tetrahydrolycopene in the evolved extended desaturation pathway of I14.

The biosynthetic pathway consisting of GGDP synthase (*crtB*<sub>EU</sub>), phytoene synthase (*crtE*<sub>EU</sub>), and either wild-type phytoene desaturase (*crtI*<sub>EU</sub>) or mutant I14 was extended with the genes for the lycopene cyclase (*crtY*) from *E. uredovora* or *E. herbicola* by cloning the desaturase genes into pAC-*crtE*<sub>EU</sub>-*crtB*<sub>EU</sub> to yield pAC-*crtE*<sub>EU</sub>-*crtB*<sub>EU</sub>-*crtI*<sub>EU</sub>/I14 and complementation of *E. coli* pAC-*crtE*<sub>EU</sub>-*crtB*<sub>EU</sub>-*crtI*<sub>EU</sub>/I14 with pUC-*crtY*<sub>EU</sub> or pUC-*crtY*<sub>EH</sub>. *Escherichia coli* cells expressing wild-type desat-

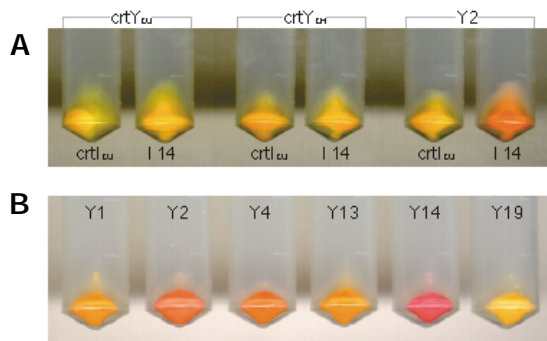
## RESEARCH ARTICLES



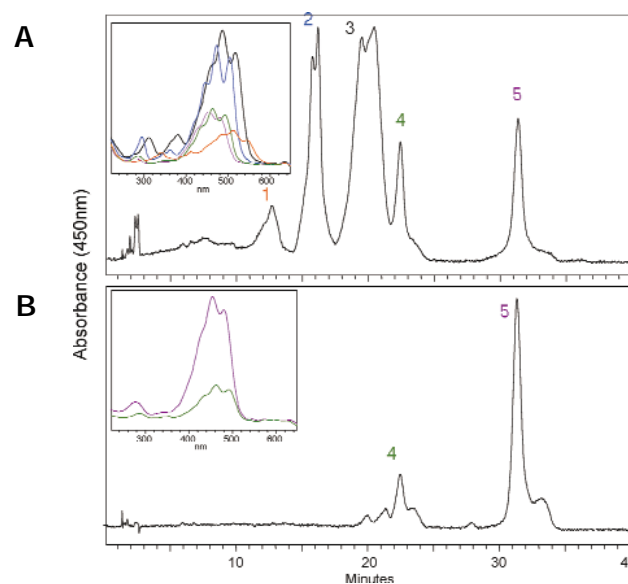
**Figure 2.** HPLC analysis of carotenoid extracts of *E. coli* transformants carrying plasmids pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>* and (A) pUC-*crtI<sub>EU</sub>* expressing the wild-type phytoene desaturase; (B) pUC-I14 expressing desaturase mutant I14; (C) pUC-I25 expressing desaturase mutant I25. The following carotenoids were identified: peak 1, 3, 4, 3', 4'-tetrahydrolycopene ( $\lambda_{\max}$  = 480 510 540 nm); peak 2, lycopene ( $\lambda_{\max}$  = 444 470 502 nm); peak 3, neurosporene ( $\lambda_{\max}$  = 415 440 468 nm); peak 4,  $\zeta$ -carotene ( $\lambda_{\max}$  = 378 400 425 nm). Double peaks indicate different geometrical isomers. Insets: recorded absorption spectra of individual HPLC peaks. Corresponding carotenoid extracts are shown. Results for pUC-*crtI<sub>EH</sub>* were similar to pUC-*crtI<sub>EU</sub>*.

urase *crtI<sub>EU</sub>* on pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-crtI<sub>EU</sub>* together with the lycopene cyclases *crtY<sub>EU</sub>* or *crtY<sub>EH</sub>* on pUC-*crtY<sub>EU</sub>* or pUC-*crtY<sub>EH</sub>*, respectively, synthesized predominantly  $\beta,\beta$ -carotene from lycopene and turned bright yellow-orange (Fig. 3A). A less-polar carotenoid with a spectrum typical for  $\beta$ -zeacarotene, the monocyclic product derived from neurosporene, was also produced (data not shown). In contrast, *E. coli* pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-I14* expressing I14 desaturase together with the lycopene cyclases only synthesized  $\beta,\beta$ -carotene (not shown) and developed a bright orange color (Fig. 3A). Neither 3,4,3',4'-tetrahydrolycopene nor cyclization products of its precursor 3,4-didehydrolycopene are synthesized, suggesting that lycopene (the precursor to 3,4-didehydrolycopene and 3,4,3',4'-tetrahydrolycopene) is a good substrate for the wild-type cyclases. Desaturase variant I14 appears to have higher desaturation activity than the wild-type enzyme, since no neurosporene accumulates that can be cyclized to  $\beta$ -zeacarotene.

To access the cyclization products of the extended desaturase pathway, then, a library of lycopene cyclases was created by shuffling the genes *crtY<sub>EU</sub>* and *crtY<sub>EH</sub>*. This library was used to transform *E. coli* cells harboring pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-I14*. Among ~4,500 clones screened, 20% were pink as a result of inactivation of the cyclase. Twenty-five colonies with colors different from wild type were selected (Fig. 3B), including some that were orange-red to purple-red, indicating the possible cyclization of 3,4-didehydrolycopene. The selected clones accumulated different ratios of lycopene,



**Figure 3.** Cell pellets of *E. coli* transformants expressing wild-type and mutant cyclases. (A) JM109 carrying plasmid pUC-*crtY<sub>EU</sub>* or pUC-*crtY<sub>EH</sub>* together with pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-crtI<sub>EU</sub>* or pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-I14*. (B) JM109 transformants carrying pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-I14* and various cyclase mutants.



**Figure 4.** HPLC analysis of carotenoid extracts of *E. coli* transformants carrying plasmids (A) pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-I14* and pUC-*crtY<sub>EH</sub>* expressing desaturase mutant I14 together with cyclase mutant Y2 and (B) pAC-*crtE<sub>EU</sub>-crtB<sub>EU</sub>-crtI<sub>EU</sub>* and pUC-*crtY<sub>EU</sub>* expressing wild-type desaturase together with cyclase mutant Y2. The following carotenoids were identified: peak 1, 3, 4, 3', 4'-tetrahydrolycopene ( $\lambda_{\max}$  = 480 510 540 nm;  $M^+$  at  $m/e$  = 532.4); peak 2, lycopene ( $\lambda_{\max}$  = 444 470 502 nm,  $M^+$  at  $m/e$  = 536.4); peak 3, torulene ( $\lambda_{\max}$  = 454 480 514 nm,  $M^+$  at  $m/e$  = 534.5); peak 4,  $\beta,\psi$ -carotene ( $\lambda_{\max}$  = 435 450 478 nm,  $M^+$  at  $m/e$  = 536.4); peak 5,  $\beta,\beta$ -carotene ( $\lambda_{\max}$  = 425 450 478 nm,  $M^+$  at  $m/e$  = 536.4). Double peaks represent different geometrical isomers. Insets: recorded absorption spectra of individual peaks.

3,4,3',4'-tetrahydrolycopene and  $\beta,\beta$ -carotene, whereas clones expressing wild-type enzymes exclusively formed  $\beta,\beta$ -carotene.

One clone Y2 appeared bright red compared to the yellow-orange color of the wild type (Fig. 3A); its extract showed a marked absorption maximum of 480 nm. Analysis by HPLC revealed small amounts of the acyclic carotenoids lycopene and 3,4,3',4'-tetrahydrolycopene and the lycopene cyclization products  $\beta,\beta$ -carotene and  $\beta,\psi$ -carotene, as well as a new, major carotenoid (Fig. 4A). The absorption maxima<sup>23</sup>, mass, and polarity of this new product show it to be torulene, the cyclization product of 3,4-didehydrolycopene. Expression of the Y2 cyclase together with the wild-type desaturase, in contrast, resulted in the synthesis of monocyclic  $\beta,\psi$ -carotene and dicyclic  $\beta,\beta$ -carotene, and no torulene (Fig. 4B). Sequence analysis of mutant Y2 revealed two amino acid changes, R330H and F367S, in the sequence of the *E. uredoovora* cyclase and no recombination. Neither mutation is located in motifs conserved among various cyclases<sup>30</sup>.

Extension of the pathway to 3,4-didehydrolycopene with a functional cyclase was accomplished by DNA shuffling, leading to the first reported synthesis of torulene in *E. coli*. Torulene is not produced by the organisms from which the biosynthetic genes were obtained, and its biosynthesis in red yeasts such as *Rhodotorula* and *Phaffia*<sup>13</sup> occurs by a different metabolic route. Analysis of pigment accumulation in *Rhodotorula glutinis* and *Phaffia rhodozyma* suggested biosynthesis of torulene from  $\beta$ -zeacarotene, the monocyclic product derived from neurosporene, through desaturation of the 7,8-dihydro—end group rather than cyclization of 3,4-didehydrolycopene<sup>17,32</sup>. The enzyme catalyzing this desaturation has not yet been characterized. Thus the in vitro evolution has extended the biosynthetic pathway with a catalytic function not available from a natural source.

Molecular pathway breeding by assembling genes into a metabolic pathway and evolving key enzymes is an efficient strategy for the synthesis of new compounds in *E. coli*. In vitro evolution allowed us to engineer the catalytic properties of two enzymes for which we have no three-dimensional structures and very little knowledge of the catalytic mechanisms. With additional biosynthetic genes and further evolution, we should be able to produce yet more novel carotenoids in *E. coli*. Because many new biosynthetic pathways can be created in a single molecular breeding experiment, the resulting organisms represent potentially interesting natural product libraries for discovery of new biologically active compounds.

### Experimental protocol

**Cloning and culture growth.** Genes for GGDP synthase ( *crtE<sub>EU</sub>*), phytoene synthase ( *crtB<sub>EU</sub>*), phytoene desaturase ( *crtI<sub>EU</sub>*,  *crtI<sub>EH</sub>*), and lycopene desaturase ( *crtY<sub>EU</sub>*,  *crtY<sub>EH</sub>*) were amplified from genomic DNA of *Erwinia uredovora* (*Pantoea ananatis* DSM 30080) and *Erwinia herbicola* EhoI (*Pantoea ananatis* DSM 30071) (GenBank accession codes: D90087, M87280, M99707) using a 5'-polymerase chain reaction (PCR) primer, which contained at its 5' end a *Xba*I site ( *crtE<sub>EU</sub>*,  *crtB<sub>EU</sub>*,  *crtI<sub>EU</sub>*,  *crtI<sub>EH</sub>*) or a *Eco*RI site ( *crtY<sub>EU</sub>*,  *crtY<sub>EH</sub>*), followed by the sequence 5'-AGG AGG ATT ACA AAA TG-3', providing a Shine-Dalgarno sequence (underlined) and a start codon (bold), and a 3'-PCR primer containing at its 5' end a *Eco*RI site ( *crtE<sub>EU</sub>*,  *crtB<sub>EU</sub>*,  *crtI<sub>EU</sub>*,  *crtI<sub>EH</sub>*) or a *Nco*I site ( *crtY<sub>EU</sub>*,  *crtY<sub>EH</sub>*). PCR products were then cloned into pUC19, which has been modified by deleting the *lacZ*-fragment and introducing a new multiple cloning site (5'-*Xba*I-*Sma*I-*Eco*RI-*Nco*I-*Not*I), thereby changing the operator sequence to facilitate constitutive expression. GGDP-synthase ( *crtE<sub>EU</sub>*) and phytoene desaturase ( *crtB<sub>EU</sub>*) were subcloned into the *Bam*HI site ( *crtB<sub>EU</sub>*) or *Clal* site ( *crtE<sub>EU</sub>*) of pACmod (pACYC184 devoid of the *Xba*I site) by amplification of the genes together with the *lac*-promoter using primer that introduce at both sites a *Bam*HI site or *Clal* site, respectively. The two reading frames face each other in the resulting plasmid pAC- *crtE<sub>EU</sub>*- *crtB<sub>EU</sub>*. Similarly, phytoene desaturase (wild type or mutant) was subcloned from pUC into the *Hind*III site of pAC- *crtE<sub>EU</sub>*- *crtB<sub>EU</sub>* to give pAC- *crtE<sub>EU</sub>*- *crtB<sub>EU</sub>*- *crtI<sub>EU</sub>*/ *crtI<sub>EH</sub>*/114 where both genes  *crtE<sub>EU</sub>* and phytoene desaturase have the same orientation. For carotenoid biosynthesis, transformed *E. coli* JM101 or the recombination-deficient strain JM109 (for stable propagation of mutant I14 during carotenoid biosynthesis) were cultivated for 24 h at 28°C in the dark in Luria-Bertani (LB) medium (500 ml medium in a 1 L flask) supplemented with 50  $\mu$ g ml<sup>-1</sup> chloramphenicol and 50  $\mu$ g ml<sup>-1</sup> carbenicillin.

**Analysis of carotenoids.** Wet cells (300 mg) were extracted with 1 ml acetone and reextracted with an equal volume of hexane after addition of 1/5 volume water. A 20  $\mu$ l aliquot of extract was applied to a Spherisorb ODS 2 column (250  $\times$  4.6 mm, 5  $\mu$ m; Waters, Milford, MA), and eluted with acetonitrile-isopropanol (99:1) at a flow-rate of 2 ml min<sup>-1</sup> using an Alliance HPLC system equipped with a photodiode array detector from Waters. Mass spectra were obtained with a Hewlett-Packard (Agilent Technologies, Palo Alto, CA) Series 1100 LC/MSD coupled with atmosphere pressure chemical ionization (APCI) interface.

**DNA shuffling and library screening.** A library of phytoene desaturase variants was created by DNA shuffling of the genes  *crtI<sub>EU</sub>* and  *crtI<sub>EH</sub>* from *E. uredovora* and *E. herbicola*, respectively, using the protocol from Stemmer<sup>5</sup>. The final amplification products were ligated into pUC and transformed into phytoene-producing *E. coli* JM101 cells containing pAC- *crtE<sub>EU</sub>*- *crtB<sub>EU</sub>*. Transformants were plated on LB plates supplemented with 50  $\mu$ g ml<sup>-1</sup> carbenicillin and chloramphenicol. After 24 h of incubation at 30°C in the dark, colonies were replicated using a nitrocellulose membrane and transferred onto fresh LB plates. Colonies were screened visually for color variants after an additional 12 h (or

until color developed) incubation. Overnight cultures (5 ml LB) were inoculated with selected colonies for analysis of carotenoid synthesis. A library of lycopene cyclase variants was created by shuffling  *crtY<sub>EU</sub>* and  *crtY<sub>EH</sub>* from *E. uredovora* and *E. herbicola*, respectively. After ligation into pUC, the library was used to transform *E. coli* JM109 cells harboring plasmid pAC- *crtE<sub>EU</sub>*- *crtB<sub>EU</sub>*-114.

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