Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1761 (2006) 235–246
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Biosynthesis of novel carotenoid families based on unnatural carbon backbones: A model for diversification of natural product pathways

Alexander V. Tobias [1], Frances H. Arnold *

Division of Chemistry and Chemical Engineering, California Institute of Technology 210-41, 1200 E. California Blvd., Pasadena, CA 91125, USA

Received 4 November 2005; received in revised form 3 January 2006; accepted 4 January 2006
Available online 30 January 2006

Abstract

We show that the C_{40} carotenoid desaturase CrtI from *Pantoea ananatis* (*Erwinia uredovora*) is capable of desaturating unnaturally long C_{45} and C_{50} carotenoid backbones in recombinant *E. coli*. Desaturation step number in these pathways is not very specific, and at least ten new C_{45} and C_{50} carotenoids were synthesized. We also present evidence for a novel asymmetric C_{40} backbone formed by the condensation of farnesyl diphosphate (C_{15}PP) with farnesylgeranyl diphosphate (C_{25}PP), and the subsequent desaturation of this backbone by CrtI in an atypical manner. Under some conditions, the C_{40}, C_{45}, and C_{50} carotenoid backbones synthesized in *E. coli* were monohydroxylated; their desaturation by CrtI in vitro led to yet more novel carotenoids. Challenging CrtI with larger-than-natural substrates in vivo has allowed us to show that this enzyme regulates desaturation step number by sensing the end groups of its substrate. Analysis of the mechanisms by which chemical diversity is generated and propagated through the nascent pathways provides insight into how natural product diversification occurs in nature.

Keywords: Molecular evolution; Carotenoid biosynthetic pathway

1. Introduction

Carotenoids are lipid pigments that play vital roles in key biological processes such as photosynthesis, vision, and the quenching of free radicals and singlet oxygen [1]. The ~700 carotenoids identified in nature are biosynthesized from two major pathways. More than 95% are based on the symmetric C_{40} backbone phytoene, formed by condensation of two molecules of geranylgeranyl diphosphate (GGPP, C_{20}PP, see Fig. 1). The C_{40} pathway, in addition to being the most diverse, is also the most widespread, appearing in thousands of species of bacteria, archaea, algae, fungi, and plants. A separate, C_{30} pathway that begins with the fusion of two molecules of farnesyl diphosphate (FPP, C_{15}PP, see Fig. 1) accounts for the remainder of natural carotenoid diversity. C_{30} carotenoids are known in only a small group of bacteria such as *Staphylococcus*, *Streptococcus*, *Methylobacterium*, and *Heliobacterium* species [2].

In recent years, carotenoid pathways have served as convenient and powerful multi-enzyme platforms for in vitro evolution experiments [3–14]. (See [15] for a recent review of this topic.) Biosynthetic pathways leading to dozens of novel carotenoids have been established by co-expression of biosynthetic genes in combinations not seen in nature, by mutation of the genes, or by a combination of the two. We are interested in how quickly and to what extent natural product biosynthetic pathways discover new chemical diversity. Examining natural pathways provides only a very late glimpse of the processes of pathway diversification and divergence. To identify the early genetic modifications that give rise to new metabolites, one must capture pathways in the earliest stages of emergence. Only then can the impact of specific mutations or new enzyme combinations on the products of the evolving pathways be discerned.

Previously, our laboratory reported the biosynthesis of novel C_{45} and C_{50} carotenoid backbones in recombinant *E. coli* expressing the Y81A mutant of the farnesyl diphosphate (C_{15}PP) synthase from *Bacillus stearothermophilus*, BstFPS$_{Y81A}$ [16]

* Corresponding author. Tel.: +1 626 395 4162; fax: +1 626 568 8743.
E-mail address: frances@cheme.caltech.edu (F.H. Arnold).
[1] Current address: DuPont Central Research and Development, Experimental Station Box 80323, Wilmington, DE 19880, USA.

1388-1981/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbalip.2006.01.003

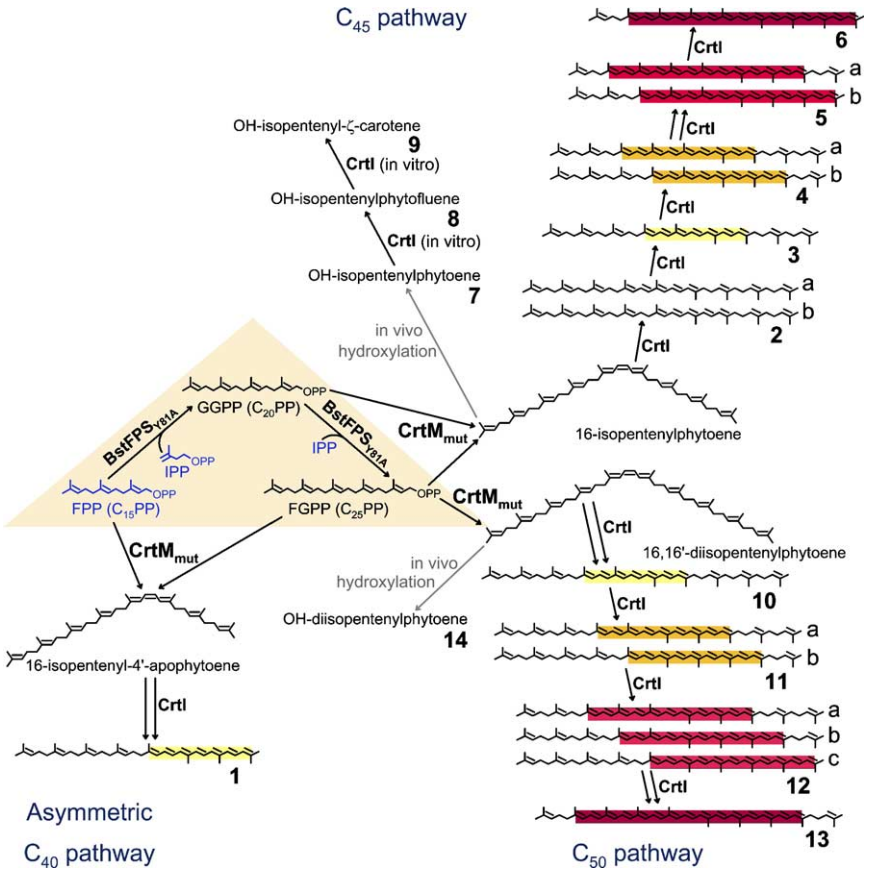

Fig. 1. Biosynthetic routes to the novel carotenoids reported in this work. *E. coli* cells expressing $BstFPS_{Y81A}$, $CrtM_{F26L}$ or $CrtM_{F26A,W38A}$ ($CrtM_{mut}$), and CrtI from *P. ananatis* synthesized novel desaturated C_{45}, C_{50}, and asymmetric C_{40} carotenoids. Colored boxes highlighting carotenoid chromophores depict the approximate color of the molecule in white light. Carotenoids reported for the first time are labeled with numbers (and letters for different desaturation isomers) used throughout the text and other figures. Names for the numbered structures are listed in Table S1 (supplementary material). Double arrows signify two desaturation steps. Carotenoid 2 was only detected from in vitro desaturation of 16-isopentenylphytoene. Carotenoid backbones are depicted as the 15*Z* isomer synthesized by these bacterial carotenoid synthases. Prenyl diphosphate precursors endogenous to *E. coli* are depicted in blue. IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FGPP, farnesylgeranyl diphosphate.

and the F26A, W38A double mutant of the C_{30} carotenoid synthase CrtM from *Staphylococcus aureus* [17], $CrtM_{F26A,W38A}$ [14]. $BstFPS_{Y81A}$ synthesizes a mixture of $C_{15}PP$, $C_{20}PP$, and farnesylgeranyl diphosphate (FGPP, $C_{25}PP$) precursors. The next enzyme, $CrtM_{F26A,W38A}$, has an expanded substrate and product range compared to wild-type CrtM, which can only synthesize C_{30} and C_{35} carotenoid backbones [12,13]. When $BstFPS_{Y81A}$ and $CrtM_{F26A,W38A}$ were co-expressed in *E. coli*, a mixture of C_{35}, C_{40}, C_{45}, and C_{50} carotenoid backbones was produced [14].

Here, we report the subsequent desaturation of these unnatural C_{45} and C_{50} carotenoid backbones by the carotenoid desaturase CrtI from *Pantoea ananatis* (*Erwinia uredovora*) [18], leading to an array of novel C_{45} and C_{50} carotenoid pigments. We found that production of specific desaturation products and absence of others provides insight into the mechanism by which CrtI regulates desaturation step number. We also present evidence for a novel "asymmetric" C_{40} carotenoid pathway beginning with the condensation of $C_{15}PP$ and $C_{25}PP$. Finally, we show that large carotenoid backbones are further diversified in an unusual manner by in vivo hydroxylation under certain conditions, and that some of these hydroxylated backbones can serve as substrates for CrtI. These results illustrate some of the means by which chemical diversity can be generated and propagated and suggest an explanation for the extensive branching and immense product diversity of many natural product pathways.

2. Materials and methods

2.1. Genes and plasmids

Genes are listed in transcriptional order in the names of all the plasmids in this report. Plasmids based on the high-copy pUCmodII vector [10,14] are designated by names beginning with "pUCmodII." The carotenoid biosynthetic genes are expressed as an operon under the control of a single *lac* promoter with no *lac* operator. In plasmids pUCmodII-$crtM_{F26L}$-$bstFPS_{Y81A}$ and pUCmodII-$crtM_{F26A,W38A}$-$bstFPS_{Y81A}$, a variant of the C_{30} carotenoid synthase gene *crtM* from *Staphylococcus aureus* ($crtM_{F26L}$ or $crtM_{F26A,W38A}$ [12,14,19]) is flanked by *Xba*I and *Xho*I restriction sites. $bstFPS_{Y81A}$, which encodes the Y81A mutant of the farnesyl diphosphate synthase from *Bacillus stearothermophilus* [14,16], is flanked by *Eco*RI and *Nco*I restriction sites and directly follows the *crtM* variant. Plasmids pUCmodII-$crtM_{F26L}$-*crtI*-$bstFPS_{Y81A}$ and pUCmodII-$crtM_{F26A,W38A}$-*crtI*-$bstFPS_{Y81A}$ additionally contain the gene encoding the carotenoid desaturase CrtI from *Pantoea ananatis* ("*Erwinia uredovora*"), which is inserted between the *crtM* and *bstFPS* genes and is flanked by *Xho*I and

*Eco*RI restriction sites. Plasmid pUCmodII-*crtI*, used to express *P. ananatis* CrtI alone for in vitro desaturation experiments, contains *crtI* flanked by *Eco*RI and *Nco*I restriction sites.

Plasmid pUC18m-*bstFPS*$_{Y81A}$, based on the high-copy vector pUC18m [12], contains the *bstFPS*$_{Y81A}$ gene flanked by *Xba*I and *Xho*I restriction sites and expressed under the control of a *lac* promoter and operator.

Plasmids based on the medium-copy vector pACmod (pACYC184 with the *Xba*I site removed) [10] were constructed by insertion of a fragment containing the entire operon from a pUC18m-based plasmid (including the *lac* promoter and operator) into pACmod. In plasmid pAC-*crtM*$_{F26A,W38A}$, *crtM*$_{F26A,W38A}$ is inserted between *Xba*I and *Xho*I restriction sites. In pAC-*crtM*$_{F26A,W38A}$-*crtI*, *crtI* from *P. ananatis* is also present and is flanked by *Xho*I and *Apa*I sites.

In all plasmids listed above, an optimized Shine–Dalgarno ribosomal binding sequence (AGGAGG) followed by eight spacer nucleotides is situated directly upstream of each gene's start codon. Even for the plasmids with *lac* operator sequences, leaky transcription was sufficient to effect the expression of carotenogenic genes. Therefore, all experiments described in this report were performed without IPTG (isopropyl-β-D-thiogalactopyranoside) induction.

2.2. Bacterial cultures and carotenoid extraction

E. coli XL1-Blue cells (Stratagene) were transformed with plasmid DNA and plated on Luria–Bertani (LB)-agar plates supplemented with 50 mg/l of each appropriate antibiotic (carbenicillin for pUC-based plasmids, chloramphenicol for pAC-based plasmids). Colonies were usually visible after 12 h at 37 °C, at which point 2-ml precultures of LB+antibiotics (50 mg/l each) were inoculated with single colonies and shaken at 37 °C and 250 rpm for 12–18 h. One milliliter of preculture was inoculated into 1 l of Terrific broth (TB) + antibiotics (50 mg/l each) in a 2.8-l Erlenmeyer flask. One-liter cultures were shaken at 28 °C and 250 rpm for 48 h. Optical densities at 600 nm of 1-l cultures ($OD_{600\ nm}$) were measured after 10-fold dilution into fresh TB medium. Dry cell masses were determined from $OD_{600\ nm}$ values using a calibration curve generated for XL1-Blue cultures.

Cells were pelleted by centrifugation at 2500×*g* for 15 min. For quantification of total carotenoid titers, a known amount of β,β-carotene (Fluka/Sigma) was added to pellets as an internal standard before extraction. Cell lysis and initial carotenoid extraction was achieved by addition of 100 ml of acetone supplemented with 30 mg/l of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT; Sigma) followed by vigorous shaking. After this point, to avoid difficulties with mass spectrometry (MS), contact with plasticware was avoided and only glassware was used. After filtration and concentration to ~5 ml under a stream of N_2, lipids including carotenoids were partitioned twice into 8 ml of hexanes and then concentrated to ~4 ml under a stream of N_2. This extract was washed 5 times with 2 ml of deionized water, dehydrated with anhydrous $MgSO_4$, filtered, and evaporated to dryness under a stream of N_2. After dissolution of the extract into 2 ml of acetone+BHT (30 mg/l), a significant amount of fatty material usually precipitated on the sides of the vial. The mixture was sonicated at 40 °C for 10–20 min. to dissolve carotenoids trapped by the precipitate and was then filtered and evaporated to dryness under a stream of N_2. Dried extracts were stored under argon gas at −20 °C. Extracts were dissolved into 0.1–1 ml of acetonitrile prior to separation and analysis.

2.3. Separation and analysis of carotenoids

The above extracts dissolved in acetonitrile were injected onto an Xterra MS C_{18} column (3.0×150 mm, 3.5-μm particles; Waters) outfitted with a corresponding guard cartridge using an Alliance 2690 HPLC system (Waters) equipped with a photodiode array detector (PDA) set to 1.2-nm resolution. For isocratic elution, the mobile phase was acetonitrile:isopropanol 93:7 (vol/vol) and the flowrate was 0.4 ml/min. For more rapid elution of highly non-polar C_{45} and C_{50} carotenoids, a gradient method with the same flowrate was employed: 0–35 min, acetonitrile:isopropanol 93:7; 35–37 min, linear gradient to acetonitrile:isopropanol 50:50; 37–50 min, acetonitrile:isopropanol 50:50. Individual carotenoids were quantified by comparing their peak areas (determined at the wavelength of maximum absorption) to a calibration curve generated using known amounts of β,β-carotene and then multiplying by the ratio of molar extinction coefficients ($\varepsilon_{\beta,\beta\text{-carotene}}/\varepsilon_{\text{sample carotenoid}}$) [20]. The calibration curve and internal standard peak areas enabled the computation of total carotenoid titers from the HPLC data.

Fractions of the HPLC eluent containing separated carotenoids were collected and evaporated to dryness under N_2. Mass spectra were obtained using either an 1100 Series HPLC–PDA–MS system (Hewlett Packard/Agilent) equipped with an atmospheric pressure chemical ionization (APCI) interface or a Finnigan LCQ mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Electron). Purified carotenoids were dissolved in either hexanes (for APCI–MS) or methanol:dichloromethane 85:15 (vol/vol) (for ESI–MS) and flow-injected into the mobile phase, which was the same as the solvent used to prepare the sample.

Carotenoids were identified and the putative structures of novel carotenoids were assigned based on their HPLC retention times, UV-visible spectra, and mass spectra.

2.4. Acetylation of hydroxylated carotenoid backbones

To confirm the presence of a hydroxy group on carotenoid backbones, the acetylation protocol suggested by Eugster [21] was followed with slight modification. Briefly, 5–10 nmol of a hydroxylated carotenoid backbone was dissolved in 0.5 ml of pyridine (dried over BaO powder). Fifty microliters of acetic anhydride was then added, and the reactions were carried out at room temperature for 1 h. The reactions were terminated by addition of 4 ml of deionized water, and carotenoids were extracted by partitioning twice with 1 ml of hexanes. After evaporation of the solvent under a stream of N_2, the product mixture was analyzed and separated by HPLC as described above. The product and unreacted fractions were dried under N_2, dissolved in hexanes, and analyzed by APCI–MS as described above.

2.5. In vitro desaturation of carotenoid backbones

Cell lysate of *E. coli* cultures expressing CrtI was prepared for in vitro desaturation reactions as follows. 150-ml cultures of XL1-Blue(pUCmodII-*crtI*) were shaken in TB medium supplemented with carbenicillin (50 mg/l) at 28 °C and 113 rpm in upright 175 cm^2 tissue culture flasks (BD Falcon) until they reached an $OD_{600\ nm}$ of 5–6 (~48 h). Cells were centrifuged at 2500×*g* for 15 min, and each pellet from a 150-ml culture was resuspended in 20 ml of Tris–HCl buffer (50 mM, pH 8) containing 1 mM phenylmethylsulfonylfluoride (PMSF). Cells were lysed by French press, and the lysate was stored at −20 °C in 1-ml aliquots until use.

Carotenoid backbone substrates for in vitro desaturation reactions were separated and purified as described above from cultures of XL1-Blue (pUCmodII-*crtM*$_{F26L}$-*bstFPS*$_{Y81A}$), XL1-Blue(pUCmodII-*crtM*$_{F26A,W38A}$-*bstFPS*$_{Y81A}$), or XL1-Blue(pUC18m-*crtE-crtB*) (synthesizing authentic phytoene only [12]). After dissolution in a known amount of hexanes, carotenoid backbones were quantified by their absorbance at 286 nm using a Cary 100 Bio UV-visible spectrophotometer (Varian). For each in vitro desaturation reaction, 10 nmol of a carotenoid backbone was dissolved in 10 μl of acetone and added to a thawed 1-ml aliquot of XL1-Blue(pUCmodII-*crtI*) lysate supplemented with additional flavin adenine dinucleotide (FAD, 1 mM) and $MgCl_2$ (4 mM). Reaction mixtures were then incubated at 30 °C with gentle end-over-end rotation for 12–18 h.

To extract carotenoids from in vitro reactions for HPLC analysis, the reaction mixtures were centrifuged at 14,000×*g* for 5 min and the pellets were separated from the supernatants. Pellets were extracted with 1 ml of acetone+BHT (30 mg/l), thoroughly vortexed, and then filtered. Carotenoids were extracted from the aqueous supernatants by partitioning twice with 1 ml of hexanes. The hexane and acetone extracts of each reaction mixture were pooled and evaporated to dryness under a stream of N_2. Carotenoids were then dissolved in 150 μl of acetonitrile and analyzed by HPLC as described above.

2.6. Iodine-catalyzed photoisomerization

To enrich mixtures of *E*- and *Z*-carotenoids in all-*E* (*trans*) isomers or verify the all-*E* configuration of specific carotenoids, carotenoids were subjected to

Table 1
Properties of novel desaturated carotenoids synthesized by XL1-Blue (pUCmodII-*crtM*$_{F26A,W38A}$-*crtI*-*bstFPS*$_{Y81A}$)

Backbone	Conjugated double bonds	Retention time[a] (min)	Molecular ion (*m*/*z*)[b]	Mol%[c]	UV-visible spectrum (Fig. 2) and putative structure (Fig. 1)
C_{40}	7	19.2	540.2	5	1
C_{45}	7	30.0	608.4	0.5	3
C_{45}	9	28.5	606.2	1	4
C_{45}	13	15.6, 16.4	602.0	4	5
C_{45}	15	19	600.2	2	6
C_{50}	7	42.0	676.2	2	10
C_{50}	9	39.6, 40.4	674.2	5	11a
C_{50}	9	38.0	674.2	0.5	11b
C_{50}	11	35.9	672.3	3	12a
C_{50}	11	34.8	672.3	2	12b/c
C_{50}	15	20.3, 21.4	668.1	5	13

[a] Using the column and gradient method described in Materials and methods. Multiple times refer to *E*/*Z* isomers of the same carotenoid.
[b] Determined by electrospray mass spectrometry. Carotenoids underwent direct electrochemical oxidation to give the radical molecular cation $[M\cdot]^+$.
[c] Approximate, of all carotenoids detected in the culture extract by HPLC. Unmetabolized 16,16′-diisopentenylphytoene (C_{50} backbone) represented ~50 mol% of this population.

iodine-catalyzed photoisomerization following the protocol suggested by Schiedt and Liaaen-Jensen [22].

3. Results

3.1. CrtI desaturates unnatural C_{45}, C_{50}, and asymmetric C_{40} carotenoid backbones, resulting in the biosynthesis of novel carotenoids

E. coli XL1-Blue cells co-expressing BstFPS$_{Y81A}$, a CrtM variant (either the F26L single mutant CrtM$_{F26L}$ or the F26A, W38A double mutant CrtM$_{F26A,W38A}$ [14]), and wild-type CrtI from *P. ananatis* synthesize at least ten desaturated carotenoids with C_{45} or C_{50} backbones. Structures 3, 4, 5, 6, 10, 11a, 11b, 12a, 12b/c, and 13 (Fig. 1), reported here for the first time, were identified by their high-performance liquid chromatography (HPLC) retention times, UV-visible spectra, and mass spectra (Table 1 and Fig. 2). (Two-dimensional NMR spectroscopy will be required for the final structural validation of the novel carotenoids reported in this work.) Fig. 1 depicts the desaturation isomers of the C_{45} and C_{50} products whose biosynthesis is supported by the HPLC and MS data (see below). We also isolated an unusual 2-step desaturated C_{40} carotenoid that is likely based on an asymmetric C_{40} backbone (structure 1, see below). Table S1 (supplementary material) lists trivial and IUPAC-IUB semi-systematic names for the structures depicted in Fig. 1.

E. coli cultures harboring the plasmid pUCmodII-*crtM*$_{F26L}$-*crtI*-*bstFPS*$_{Y81A}$, pUCmodII-*crtM*$_{F26A,W38A}$-*crtI*-*bstFPS*$_{Y81A}$, or the plasmids pUC18m-*bstFPS*$_{Y81A}$ and pAC-*crtM*$_{F26A,W38A}$-*crtI* together synthesized mixtures of all of these novel desaturated carotenoids in different proportions and titers, depending on the expression plasmid(s). With all of these expression systems, we observed almost 100% conversion of the C_{45} carotenoid backbone 16-isopentenylphytoene to desaturated C_{45} carotenoids. Only about 25% of the C_{50} backbone 16,16′-diisopentenylphytoene was converted to desaturated products under the same conditions (Table 1).

In general, the UV-visible absorption spectra of the desaturated C_{45} and C_{50} carotenoids are hypsochromically shifted compared to those of C_{40} carotenoids with the same number of conjugated double bonds (Fig. 2: 3 and 10 vs. ζ-carotene, 4 and 11a vs. neurosporene, 12a vs. lycopene). The spectra shown in Fig. 2 are the most bathochromic of those measured before and after iodine-catalyzed photoisomerization, and most of these spectra exhibit *cis*-peaks [20] that are less or only slightly more intense than the corresponding C_{40} standard (e.g., 11a vs. neurosporene, 10 vs. ζ-carotene). We therefore hypothesize that the hypsochromic shifts in the spectra of 3, 4, 10, 11a, and 12a are not caused by a high proportion of *Z*- (*cis*) isomers, but rather by unfavorable interactions between these highly nonpolar carotenoids and the much more polar, mostly-acetonitrile solvent.

Of special interest are C_{50} carotenoids 11b and 12b/c. Compared with 11a, the former has a spectrum that is hypsochromically shifted by 11 nm at the wavelength of maximum absorption, λ_{max} (Fig. 2), elutes slightly earlier in reverse-phase HPLC, is only one-tenth as abundant, and has an identical molecular ion at *m*/*z*=674.2 (Table 1). We believe that these properties are best explained by an unusual desaturation pattern in 11b in which all three desaturation steps are on one side of the molecule (Fig. 1) (denoted as "3+0" desaturation). We are not aware of other carotenoids with a 3+0 desaturation pattern whose spectra we can compare with that of 11b. However, 7,8,11,12-tetrahydrolycopene, an isomer of ζ-carotene with a 2+0 desaturation pattern, has an absorption spectrum that is hypsochromically shifted by ~5 nm compared with that of ζ-carotene, which has a 1+1 pattern [20,23–25]. Product 12b/c has a spectrum that is hypsochromically shifted by 4 nm at λ_{max} compared with that for 12a (Fig. 2), and it elutes slightly before 12a in HPLC (Table 1). This species is only slightly less abundant than 12a and has the same molecular ion at *m*/*z*=672.3 (Table 1). These properties suggest that 12b/c is a 4-step desaturated C_{50} carotenoid with a 3+1 desaturation pattern (12b in Fig. 1). However, we cannot rule out the possibility that 12b/c has a 4+0 desaturation pattern (12c) or is a mixture of 12b and 12c.

Carotenoid 5 has the mass of a 5-step desaturated C_{45} carotenoid, and its absorption spectrum corresponds closely with that of 3,4-didehydrolycopene, which has a 3+2 desaturation pattern [20,26]. There are two possible C_{45} carotenoids with this desaturation pattern (Fig. 1), and we could not distinguish by HPLC and MS analysis whether carotenoid 5 has specific structure 5a or 5b, or represents a mixture of the two. Similarly, it is not possible to confirm whether carotenoid 4 has precise structure 4a or 4b without synthetic standards or the use of two-dimensional NMR techniques requiring up to tens of milligrams of sample [27,28].

Spectra 6 and 13 are similar to that of 3,4,3′,4′-tetradehydrolycopene with 6 desaturation steps in a 3+3

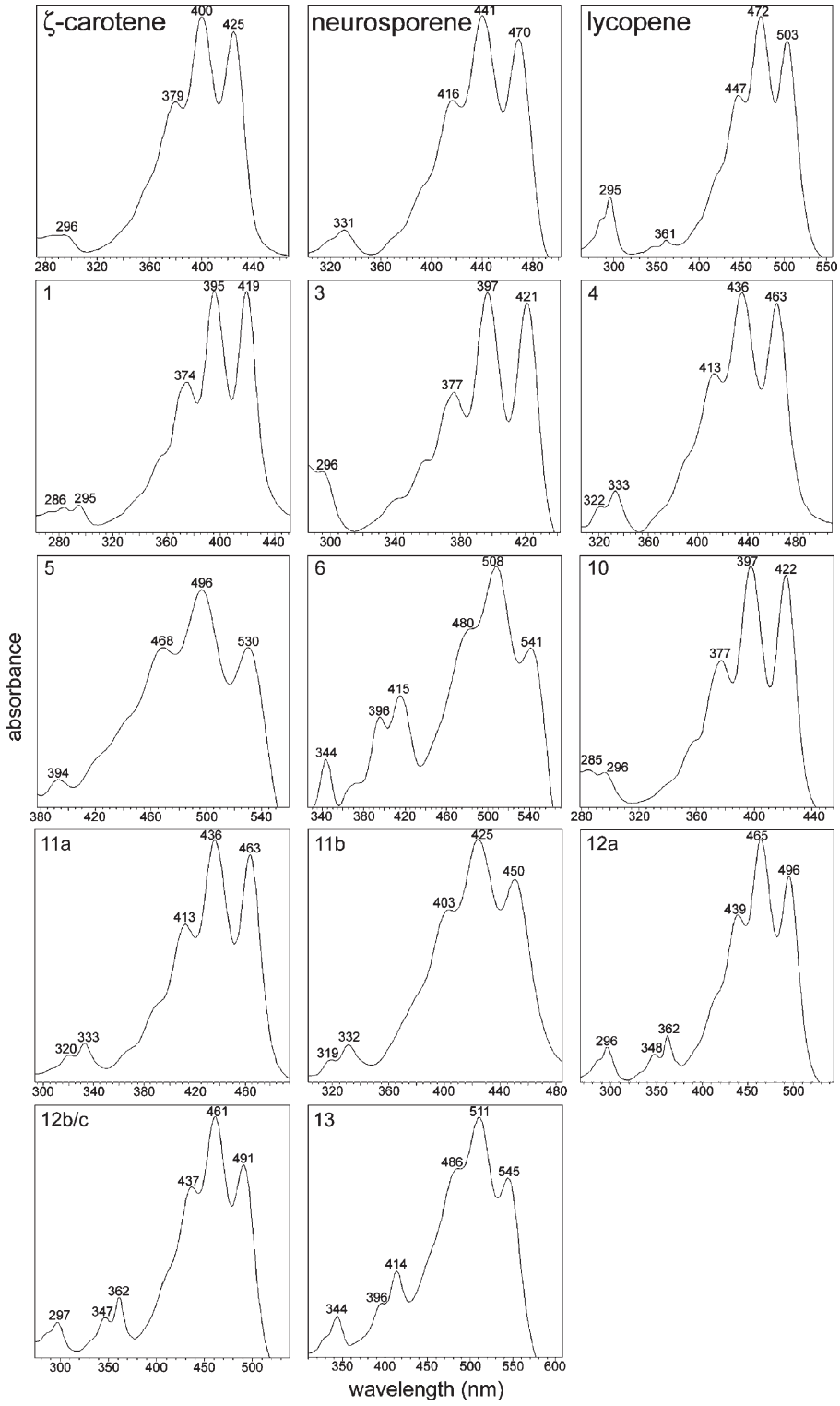

Fig. 2. UV-visible absorption spectra of novel carotenoids biosynthesized by recombinant *E. coli*. Numeric labels refer to structures depicted in Fig. 1; multiple letters indicate that the spectrum may be that of either or both structures. Spectra were measured by photodiode array directly after HPLC separation, with a mobile phase of acetonitrile:isopropanol 93:7 ([vol/vol], see Materials and methods). Spectra were taken before and after iodine-catalyzed photoisomerization; each spectrum shown is the most bathochromically shifted of the two. Absorption spectra of ζ-carotene, neurosporene, and lycopene generated by in vitro desaturation of authentic phytoene are shown for comparison. Absorption maxima are labeled with the corresponding wavelength. Table 1 lists additional properties of these molecules; Table S1 (supplementary material) lists their names.

pattern [20]. Spectrum 6 is slightly hypsochromically shifted compared to spectrum 13; this effect is likely due to a significant proportion of *Z*-isomers in our sample of carotenoid 6, evidenced by the greater relative absorbance of its cis peaks at 396 and 415 nm. Having no reason to believe that either 6 or 13 has an unusual desaturation pattern, we have

depicted these structures in Fig. 1 with symmetrical 3+3 desaturation.

Notably absent in all the extracts we analyzed of cultures expressing $BstFPS_{Y81A}$, $CrtM_{F26L}$ or $CrtM_{F26A,W38A}$, and CrtI were 4-step desaturated C_{45} and 5-step desaturated C_{50} carotenoids. We believe these observations, which may initially seem merely curious, can help to clarify the means by which this desaturase regulates step number (see Discussion).

Carotenoid 1 was initially surprising to find in the above extracts. This molecule and ζ-carotene have the same molecular ion at $m/z=540.2$ (Table 1), but compared with ζ-carotene, carotenoid 1 has a slightly longer HPLC retention time (~19 vs. ~18 min) and a UV-visible spectrum that is hypsochromically shifted by 5 nm at λ_{max} (Fig. 2). Because both molecules are C_{40} carotenoids with equal masses and virtually identical polarities, this wavelength shift cannot be due to a change in solvent–analyte interaction. Rather, we believe the shift is due to a 2+0 desaturation pattern, and that carotenoid 1 is based on an asymmetric C_{40} ($C_{15}+C_{25}$) backbone as shown in Fig. 1 (see Discussion).

3.2. *E. coli* expressing only $BstFPS_{Y81A}$ and a variant of CrtM accumulate hydroxylated carotenoid backbones

When *E. coli* XL1-Blue cells were transformed with the desaturase-free plasmids pUCmodII-*crtM*$_{F26L}$-*bstFPS*$_{Y81A}$ or pUCmodII-*crtM*$_{F26A,W38A}$-*bstFPS*$_{Y81A}$ and grown in liquid culture, they accumulated novel monohydroxylated C_{45} and C_{50} carotenoid backbones 7 and 14 as well as monohydroxylated C_{40} backbones, which may be novel depending on the location of the OH-group and whether the C_{40} backbone is symmetric or asymmetric (Fig. 1, Table 2). The biosynthesis of these hydroxylated carotenoids was confirmed by MS and chemical derivatization (Fig. 3), and their proportions relative to each other and the unmodified backbones were quite reproducible (Fig. 4). On the other hand, similar XL1-Blue cultures harboring the plasmids pUC18m-*bstFPS*$_{Y81A}$ and pAC-*crtM*$_{F26A,W38A}$ did not produce hydroxylated backbones. Some possible reasons for this disparity as well as plausible explanations for the source of hydroxylation are presented in the Discussion.

Table 2
Results of in vitro desaturation experiments with XL1-Blue(pUCmodII-*crtI*) lysate on various carotenoid backbones[a]

Backbone	Conversion (%)	Desaturation products (mol% of product mixture)			
		1-step	2-step	3-step	4-step
C_{30}	1	28	41	9	22
Phytoene[b]	19	14	13[c]	7	66
C_{40}–OH[d]	30	11	12	19	58
C_{45}	5	71 [2][e]	29 [3]	–	–
C_{45}–OH [7]	14	48 [8]	52 [9]	–	–
C_{50}	0	–	–	–	–
C_{50}–OH [14]	0	–	–	–	–

Numbers in square brackets refer to products shown in Fig. 1.

[a] Values represent the average of at least two independent experiments rounded to the closest percentage point. Coefficients of variation were less than 0.25.

[b] The native substrate of CrtI and positive control. Phytoene was purified from cultures of XL1-Blue(pUC18m-*crtE-crtB*) [12], which produces authentic phytoene only.

[c] Identified by its absorbance spectrum as ζ-carotene.

[d] Most likely predominantly OH-phytoene but may have contained some hydroxylated 16-isopentenyl-4′-apophytoene (asymmetric C_{40} backbone).

[e] This product was detected only from in vitro desaturation of 16-isopentenylphytoene (C_{45} backbone) and was not found in vivo.

Although we could not identify the specific locations of the hydroxy groups by HPLC and MS analysis, we can exclude several possible regioisomers. In vitro acetylation reactions with acetic anhydride were positive for all the hydroxylated C_{40}, C_{45}, and C_{50} backbones, with conversions above 90% in all cases, indicating that the hydroxy groups are primary or secondary [21]. Also, the propensity of the hydroxylated and acetylated carotenoids to lose water or acetate, respectively, in atmospheric pressure chemical ionization mass spectrometry (APCI–MS) (Fig. 3) is evidence that the substituents are allylic [29]. Finally, that some of these hydroxylated backbones are desaturated by CrtI in vitro (see below) suggests the OH group is far from the center of the carotenoid backbone, which is where desaturases initiate their catalytic action.

Only one C_{40} backbone fraction was seen in HPLC; likewise, hydroxylated C_{40} backbones eluted as a single peak (Fig. 5). Attempts to further separate these fractions using a linear gradient of acetonitrile:isopropanol (98:2 to 93:7 over 30 min) were not successful. However, because of the strong evidence that carotenoid 1 is based on the asymmetric C_{40} carotenoid backbone 16-isopentenyl-4′-apophytoene, we nevertheless believe that the C_{40} backbone fraction is a mixture of phytoene and 16-isopentenyl-4′-apophytoene, and that the C_{40}-OH fraction may also be a mixture of hydroxylated versions of these backbones. Indeed, the subtle structural differences between symmetric and asymmetric backbones of the same length should have minimal effects on chromatographic retention.

Cultures of *E. coli* strain HB101 carrying either plasmid pUCmodII-*crtM*$_{F26L}$-*bstFPS*$_{Y81A}$ or pUCmodII-*crtM*$_{F26A,W38A}$-*bstFPS*$_{Y81A}$ also synthesized hydroxylated C_{40}, C_{45}, and C_{50} carotenoids, but in different relative proportions compared with the XL1-Blue cultures (data not shown). Surprisingly, significant proportions of acetylated C_{40} and C_{45} carotenoid backbones (~10 and ~19 mol% of total carotenoids, respectively) were detected in cultures of HB101(pUC18m-*bstFPS*$_{Y81A}$+ pAC-*crtM*$_{F26A,W38A}$) in addition to smaller amounts of hydroxylated C_{40} and C_{45} carotenoid backbones (~2 and ~4 mol%, respectively). The in vivo-acetylated carotenoids behaved identically in HPLC and APCI-MS to their counterparts produced by in vitro derivatization of hydroxylated carotenoids with acetic anhydride. These acetylated backbones are probably formed by the reaction of hydroxylated carotenoids with acetyl-CoA, a process that is somehow promoted in HB101 cells carrying these plasmids.

3.3. In vitro desaturation experiments with CrtI

To investigate the substrate specificity of *P. ananatis* CrtI and to access new desaturated carotenoids, we carried out in

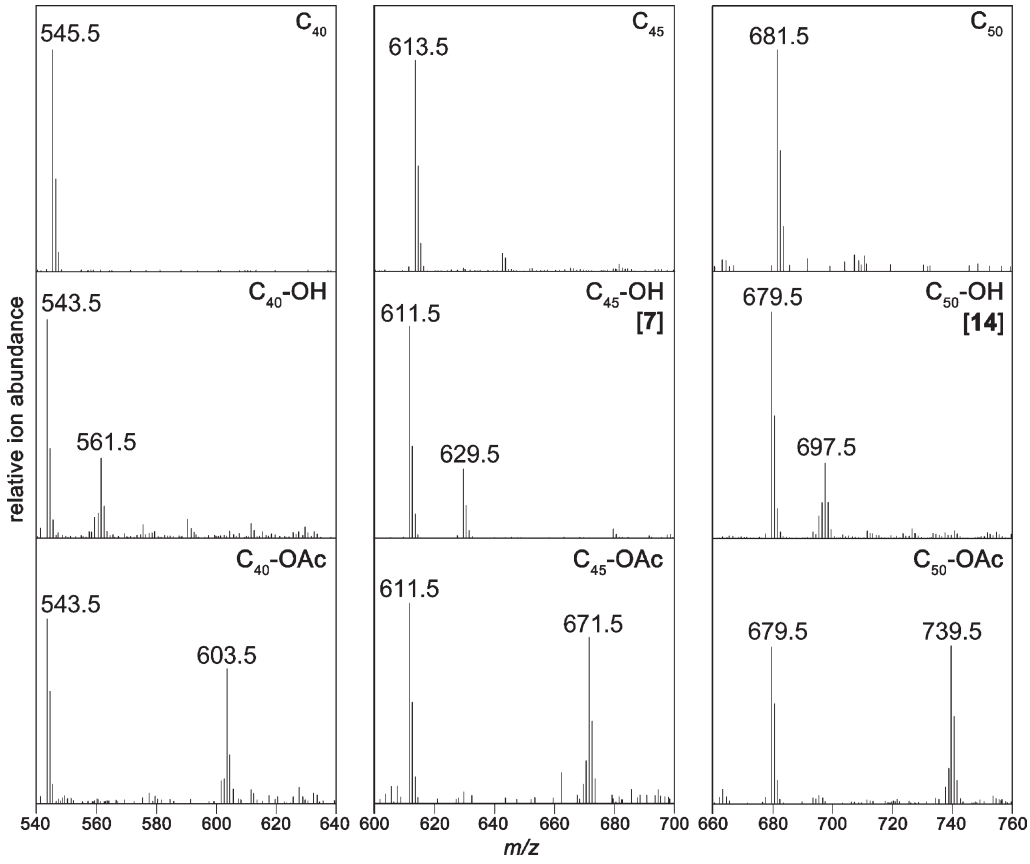

Fig. 3. Atmospheric pressure chemical ionization mass spectra of unmodified, hydroxylated, and acetylated carotenoid backbones. Unmodified carotenoid backbones ionize by protonation in APCI-MS, giving $[M+H]^+$ quasi-molecular ions (calculated monoisotopic masses for carotenoid backbones: C_{40}, 544.5 Da; C_{45}, 612.6 Da; C_{50}, 680.6 Da). Hydroxylated carotenoid backbones ionize by protonation followed by loss of water, yielding an $[(M+H)–H_2O]^+$ ion (m/z=543.5 for C_{40}–OH, 611.5 for C_{45}–OH, and 679.5 for C_{50}–OH) in addition to the $[M+H]^+$ quasi-molecular ion (m/z=561.5 for C_{40}–OH, 629.5 for C_{45}–OH, and 697.5 for C_{50}–OH). Hydroxylated backbones were acetylated by reaction with acetic anhydride. Mass spectra of the acetylated products reveal a similar mode of ionization to their hydroxylated counterparts, yielding the $[(M+H)–CH_3COOH]^+$ ion (identical to the $[(M+H)–H_2O]^+$ ions listed above) in addition to the $[M+H]^+$ quasi-molecular ion (m/z=603.5 for C_{40}–OAc, 671.5 for C_{45}–OAc, and 739.5 for C_{50}–OAc). Numbers in square brackets refer to products shown in Fig. 1.

vitro desaturation reactions in which *E. coli* lysate containing CrtI was incubated with individual carotenoid backbones. The C_{45} backbone was desaturated by *P. ananatis* CrtI in vitro to 1- and 2-step products (Table 2). The predominant C_{45} desaturation product was the 1-step carotenoid *2*, which was not detected at all in vivo. Therefore, the reduced step number displayed by CrtI in vitro allowed access to a novel carotenoid that did not accumulate in cultured cells. The C_{50} backbone diisopentenylphytoene was not desaturated in vitro, reaffirming our in vivo results showing that a substantial proportion of this backbone synthesized in *E. coli* cells remained unmetabolized by CrtI.

CrtI also desaturated hydroxylated C_{40} and C_{45} backbones to produce (at least) two more new carotenoids, 8 and 9. (The monohydroxy 1-, 2-, 3-, and 4-step desaturated C_{40} carotenoids (Table 2) may also be novel, depending on the location of the hydroxy group.) Because the precise position of the hydroxy group in the C_{45}-OH substrate 7 is unknown, we do not know the exact structures of products 8 and 9. Accordingly, we have represented these carotenoids only by name in Fig. 1. The hydroxy group does not seem to interfere with the desaturation sequence of CrtI, at least up to the first two steps. We interpret this as evidence that the OH group is located distal to the center of the molecule.

4. Discussion

4.1. Determination of desaturation step number by Pantoea CrtI

Genetic complementation experiments with heterokaryons of the fungus *Phycomyces blakesleeanus* have provided evidence for the existence of multienzyme complexes that function as assembly lines for carotenoid biosynthesis in that organism [30–33]. In these complexes, carotenoid substrates are believed to undergo stepwise chemical transformations as they are processed by one enzyme and then passed on to the next one, hence the analogy to an industrial assembly line. Therefore, phytoene undergoes four desaturation steps in *Phycomyces* because four desaturase subunits are present in that organism's carotenoid biosynthetic enzyme complexes [30]. Carotenogenic enzyme complexes are thought to be present in other organisms as well, although there are many uncertainties regarding the factors that determine the extent of carotenoid desaturation [34].

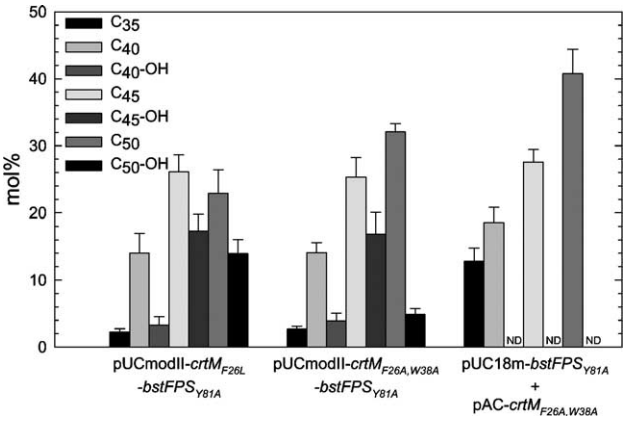

Fig. 4. Relative molar quantities of the carotenoid backbones produced by recombinant XL1-Blue cultures expressing BstFPS$_{Y81A}$ and a CrtM variant. Transformed plasmid(s) are shown on the horizontal axis. Bar heights represent the average of measurements of at least three independent cultures; error bars, standard deviations. Total carotenoid titers varied to a greater extent than relative molar quantities, approximate values were: pUCmodII-*crtM$_{F26L}$-bstFPS$_{Y81A}$*, ~100 nmol/g dry cells; pUCmodII-*crtM$_{F26A,W38A}$-bstFPS$_{Y81A}$*, ~300 nmol/g dry cells; pUC18m-*bstFPS$_{Y81A}$*+pAC-*crtM$_{F26A,W38A}$*, ~70 nmol/g dry cells. ND, not detected. The C_{40} and C_{40}–OH data series likely represent mixtures of symmetric and asymmetric C_{40} backbones that did not separate by the HPLC methods described in Materials and methods.

Our results on the in vivo desaturation of the C_{45} backbone isopentenylphytoene by *P. ananatis* CrtI provide evidence against the idea that the number of subunits in a complex determines the desaturation step number. *P. ananatis* CrtI is primarily a 4-step desaturase in the symmetric C_{40} pathway, converting phytoene to lycopene as the majority product [12,35,36]. If the primary determinant of this 4-step product specificity were the association of CrtI subunits as tetramers, we should also expect mainly 4-step products with other carotenoid backbones, assuming these substrates are accepted and processed by the complex. However, not only is this not the case in the C_{45} pathway, but 4-step C_{45} products do not accumulate at all, even though the higher step-number C_{45} products 5 (5 steps, majority product) and 6 (6 steps, second-most abundant) do accumulate (Table 1, Fig. 1).

We believe this observation is related to two others. First, in the C_{50} pathway, we observed no accumulation of 5-step products in vivo (Table 1, Fig. 1), even though 6-step C_{50} products were formed. Second, in vitro desaturation experiments with *P. ananatis* CrtI on phytoene have shown that the 3-step neurosporene is the least abundant intermediate, accumulating at low levels (Table 2) or not at all [35]. (In vivo, the enzyme leaves behind no intermediates and even catalyzes six desaturation steps on phytoene [12,36]).

These observations are all connected by a common trait shared by neurosporene and the likeliest possible 4-step C_{45} and 5-step C_{50} products: all have one ψ-end and one dihydro-ψ-end. This structural feature shared by all three disfavored products implies that *P. ananatis* CrtI has a strong propensity to avoid terminating its desaturation sequence at products with this combination of ends, regardless of the size of the carotenoid backbone substrate or the number of steps required. Therefore, CrtI appears to regulate desaturation step number by sensing its substrate's end groups, with particular preference for carotenoids with one ψ- and one dihydro-ψ-end, which are desaturated with high efficiency.

These results also shed light on the functional plasticity displayed by bacterial carotenoid desaturases in directed evolution experiments aimed at altering desaturation step number [10,13,15,37]. If a change in desaturation step number required a change in multimeric state, then converting a 4-step desaturase into a 6-step enzyme would require conversion of a tetrameric enzyme into a hexameric one. This would have to result from only a small number of mutations. While mutations can abolish the ability of a protein to form multimers, it seems much less plausible that the number of subunits in a complex could be so finely-tuned by minimal mutation. It is easier to envision how a desaturase's catalytic rate or tendency to synthesize products with particular end groups could be modified by mutation.

4.2. Evidence for a novel asymmetric C_{40} carotenoid biosynthetic pathway

We stated earlier that carotenoid 1 is likely the 2+0 desaturation product of an asymmetric C_{40} ($C_{15}+C_{25}$) carotenoid backbone, as shown in Fig. 1. Although 1 has the same mass and chromophore size as ζ-carotene, it is unlikely that *1* is ζ-carotene with the hypsochromic shift in its spectrum resulting from *Z*-isomerization. The spectrum of 1 (Fig. 2) shows the hallmarks of a majority all-*E* sample population: the cis peaks at 286 and 295 are low (~10% of λ_{max}), and the ratio of the height of the longest-wavelength absorption band (419 nm) to the absorption at λ_{max} (the so-called "III/II" ratio [20]) is 1.0. In fact, the spectrum shown for ζ-carotene in Fig. 2 is that of a sample with more *Z*-isomer content than 1. In that spectrum, III/II is only 0.83 (for all-trans ζ-carotene, III/II is between 1.0 and 1.028 [23,24]).

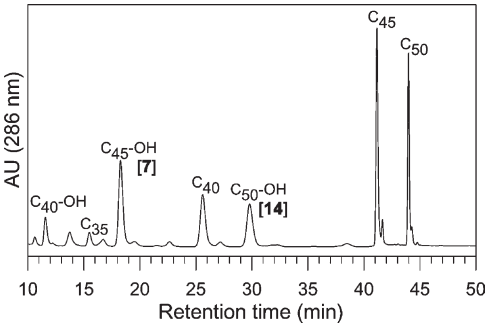

Fig. 5. HPLC trace of carotenoid backbones extracted from a culture of XL1-Blue(pUCmodII-*crtM$_{F26L}$-bstFPS$_{Y81A}$*). Carotenoids were eluted using the gradient method described in Materials and methods. The identity of each peak was confirmed by MS; the presence of monohydroxy substituents was further confirmed by acetylation and subsequent MS (see Fig. 3). The C_{40} and C_{40}-OH peaks likely represent mixtures of symmetric and asymmetric C_{40} backbones that did not separate under these elution conditions. Labeled major peaks represent 15*Z* isomers; minor peaks eluting just after major peaks represent all-*E* isomers. Numbers in square brackets refer to products shown in Fig. 1.

A 2+0 desaturation pattern also results in a hypsochromic shift of 5 nm, as shown by comparison of the spectrum of the 2+0 desaturation product of phytoene, 7,8,11,12-tetrahydrolycopene, with that of ζ-carotene, a 1+1 desaturated carotenoid [20,23–25]. However, although the absorption spectrum of 7,8,11,12-tetrahydrolycopene is strikingly similar to that of 1 [20,23–25], it is also unlikely that carotenoid 1 is 7,8,11,12-tetrahydrolycopene, because *P. ananatis* CrtI desaturates phytoene to lycopene via ζ-carotene (Table 2, Ref. [35]) and has not been shown to synthesize 7,8,11,12-tetrahydrolycopene. Furthermore, a 2-step desaturation product of phytoene like ζ-carotene or 7,8,11,12-tetrahydrolycopene is not expected to accumulate in a culture expressing *P. ananatis* CrtI. This enzyme efficiently desaturates all the available phytoene to lycopene and even 3,4,3′,4′-tetradehydrolycopene in vivo, with no intermediates being detected [12,36]. Indeed, in the same cultures in which 1 was found, lycopene was also present and was usually at least twice as abundant; tetradehydrolycopene was also detected in smaller amounts (data not shown). Finally, the presence of a 2-step C_{40} carotenoid cannot be explained by a "phytoene overload" that overwhelms CrtI, for even in engineered *E. coli* that accumulate orders of magnitude more carotenoids than XL1-Blue, this desaturase is capable of efficiently converting the vastly increased phytoene supply to lycopene, despite being expressed from a low-copy plasmid [38].

The most reasonable conclusion from the above evidence is that carotenoid 1 has an asymmetric C_{40} ($C_{15}+C_{25}$) carbon backbone that has undergone two desaturation steps on its C_{15}-side. Although we were unable to separate the asymmetric C_{40} backbone from phytoene by HPLC, it is reasonable to expect that the former backbone is made by these cultures, whose additional synthesis of C_{35}, C_{45}, and C_{50} carotenoid backbones proves that both C_{15}PP and C_{25}PP are present in the cells. Furthermore, it is not surprising that CrtI would desaturate 16-isopentenyl-4′-apophytoene differently than phytoene. Although equal in size, the former is the most asymmetric of the six possible carotenoid backbones formed from C_{15}PP, C_{20}PP, and C_{25}PP, and this likely affects the catalytic action of CrtI. When presented with the asymmetric C_{40} backbone, CrtI apparently catalyzes two desaturation steps on the C_{15}-side of the substrate (which is all that this side can accommodate) rather easily, but has trouble desaturating the C_{25}-side of the molecule; therefore, product 1 accumulates (This scenario seems more probable than 2 steps on the C_{25}-side and none on the C_{15}-side since CrtI can desaturate the C_{30} ($C_{15}+C_{15}$) backbone more efficiently than the C_{50} ($C_{25}+C_{25}$) backbone (Table 2)). We are unsure why we did not detect any asymmetric C_{40} pathway products with longer chromophores than 1. Given that CrtI can desaturate C_{45} and C_{50} backbones, it is possible that some 4–6 step asymmetric C_{40} pathway products were made but were not distinguished from symmetric C_{40} carotenoids in our analysis, leaving only product 1 to stand out because of the lack of ζ-carotene produced. Because of this possibility, we could not quantify the relative proportions of symmetric and asymmetric C_{40} carotenoids made in the cultures.

4.3. Origins of in vivo-hydroxylated carotenoids

We remain uncertain of the mechanism by which the carotenoid backbones are hydroxylated in vivo. Krubasik et al. observed some hydroxylation of the C_{50} flavuxanthin in recombinant *E. coli* cells [39] and C_{30} carotenoids synthesized in *E. coli* have also been reported to be hydroxylated by unknown processes [8,40]. Lee et al. postulated that their C_{30} diapocarotenoids were hydroxylated by free peroxy radicals present in *E. coli* membranes [8]. Like us [12], they did not observe hydroxylation of C_{40} carotenoids in *E. coli*. Albrecht et al. reported a significant amount of epoxidation and hydroxylation of phytoene in cells of the green algae *Scenedesmus acutus* whose phytoene desaturase was inhibited by the herbicide norflurazon [29].

As mentioned, XL1-Blue cultures harboring the plasmids pUC18m-*bstFPS*$_{Y81A}$ and pAC-*crtM*$_{F26A,W38A}$ produced no hydroxylated backbones (Fig. 4). These cultures also produced fewer total carotenoids than the single-plasmid cultures (see Fig. 4 caption). This expression vector-dependency of carotenoid backbone hydroxylation suggests that *E. coli* cells selectively induce carotenoid oxidation only under certain conditions. Perhaps the lack of a *lac* operator in the pUCmodII-based plasmids and the resulting high-level constitutive expression of the carotenoid biosynthetic genes therein elicits this activation in *E. coli*. Plasmids pUC18m-*bstFPS*$_{Y81A}$ and pAC-*crtM*$_{F26A,W38A}$ have their carotenogenic genes under the control of a *lac* promoter and operator, which should limit gene expression somewhat and possibly avoid the same response in the cells. Alternatively, the induction of carotenoid backbone hydroxylation might be triggered when the quantity of large carotenoid backbones in the cell membrane reaches a critical threshold.

Co-expressing CrtI along with BstFPS$_{Y81A}$ and either CrtM$_{F26L}$ or CrtM$_{F26A,W38A}$ abolished almost all backbone hydroxylation, even though C_{50} backbones were quite abundant and accounted for ~50 mol% of total carotenoids. A possible reason for this is that free radicals preferentially react with and are quenched by desaturated carotenoids, whose extended chromophores are much more reactive toward radical species compared with undesaturated carotenoid backbones [1,41–43].

4.4. Properties of nascent biosynthetic pathways

In addition to producing several novel carotenoids based on unnatural carbon scaffolds, this work has allowed us to investigate the events that resulted in the creation of three novel biosynthetic pathways. The Y81A substitution in BstFPS$_{Y81A}$ dramatically broadens the specificity of this enzyme, converting it from a strict C_{15}PP synthase into one that synthesizes a mixture of C_{15}PP, C_{20}PP, and C_{25}PP. This mutation has been studied in detail, and its effect on the enzyme's product range has been shown to result from an enlarged product elongation pocket [16]. Although capable of synthesizing products up to C_{25}PP by catalyzing two additional condensations of IPP with the growing prenyl chain, BstFPS$_{Y81A}$ also releases intermediates C_{15}PP and

C_{20}PP. This diversity is then amplified by the next enzyme in the pathway, a mutant of the *S. aureus* C_{30} carotenoid synthase CrtM.

We have previously discussed the molecular effects of mutations at F26 and W38 in CrtM [12,14,15]. A homology model based on human squalene synthase allowed us to map these residues to the site of the second half-reaction and led us to propose that wild-type CrtM can accept C_{20}PP and even C_{25}PP as substrates and form cyclopropyl intermediates from these precursors, but cannot rearrange intermediates larger than C_{35} into carotenoid backbones. When additional space is created in the rearrangement pocket by replacement of F26 and/or W38 with smaller amino acids, conversion of the larger intermediates becomes possible, resulting in the synthesis of carotenoid backbones up to C_{50}. When co-expressed with $BstFPS_{Y81A}$ in vivo, these CrtM mutants condense every possible pairwise combination among C_{15}PP, C_{20}PP, and C_{25}PP except the wild-type's combination of two molecules of C_{15}PP (a consequence of the relatively low C_{15}PP concentration combined with the intrinsically poor ability of the CrtM variants to make C_{30} carotenoids). The result is a mixture of C_{35}, symmetric ($C_{20}+C_{20}$), and asymmetric ($C_{15}+C_{25}$) C_{40}, C_{45}, and C_{50} carotenoid backbone products and, thus, amplification of the precursor diversity generated in the previous catalytic step.

The desaturase CrtI then further diversifies the five carotenoid backbones generated by the two preceding enzymes. CrtI possesses no mutations, but its inherent promiscuity allows it to accept all of the above backbones to some degree. Although CrtI efficiently and quite specifically catalyzes four desaturation steps on its native substrate phytoene in vivo, its step number in the C_{45} and C_{50} pathways is not very well defined (except for the absence of certain products discussed above), and there was no clear majority desaturation product in these pathways in vivo (Table 1). This imprecise desaturase behavior is the basis for the branching of the C_{45} and C_{50} pathways. CrtI also produces carotenoids with unusual desaturation patterns like 1, 11b, and 12b/c (Fig. 1; Table 1), indicating that unnaturally large or asymmetric substrates can cause the enzyme to carry out non-standard desaturation sequences. These results demonstrate how the unusual catalytic behavior that can result when an iterative enzyme is challenged with a new substrate can serve as a diversity-generating mechanism.

This work highlights the rapid and extensive product diversification that can result from a small number of genetic changes to a biosynthetic pathway. This diversification arises from the consecutive arrangement of three broad-specificity enzymes, two of which were evolved in the laboratory for the ability to accept new substrates and/or synthesize new products. Many of the pathways responsible for the biosynthesis of natural products in plants and microorganisms also synthesize multiple products and possess enzymes with broad specificity [44–48]. To explain this, it has been proposed that natural product pathways have evolved under selection for traits such as pathway branching and enzyme promiscuity, because such traits promote the production and retention of product diversity at minimal cost [48–51]. In this work, however, highly branched carotenoid pathways comprising laboratory-evolved promiscuous enzymes emerged without direct selection for these properties. In no instance was ability to accept multiple substrates or to synthesize several products a criterion for selection of mutants during laboratory evolution or site-directed mutagenesis experiments that resulted in mutants $BstFPS_{Y81A}$, $CrtM_{F26L}$, and $CrtM_{F26A,W38A}$ [12,14,16,19,52].

These results, complemented by directed evolution experiments on a wide range of enzymes, reveal that mutants with broadened (as opposed to shifted) specificity are the norm, even when the selection pressure requires only one particular product [53]. Enzymes that have recently evolved the ability to accept non-native substrates such as man-made antibiotics also tend to have broadened rather than shifted specificities, hence the emergence of so-called "extended-spectrum" beta-lactamases [54]. We therefore submit that the broad specificity of natural product enzymes and pathways in nature is not the outcome of positive selection for promiscuity, but rather represents the default state of enzymes and metabolic pathways in the absence of strong and sustained selective pressure to be specific. In this view, broad specificity can result from weak selection pressure for narrow specificity (genetic drift), or from selection pressures that change rapidly and hence do not allow sufficient evolutionary time for any particular narrow specificity to fully develop.

Acknowledgments

A. Tobias acknowledges support from a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship. This work was supported by grant BES-0118565 from the U.S. National Science Foundation.

We thank Mona Shahgholi and Nathan Dalleska for valuable assistance with mass spectrometry. We are also grateful to Gerhard Sandmann and George Britton for their technical advice, and to Shinichi Takaichi for providing assistance with the naming of the novel carotenoids.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbalip.2006.01.003.

References

[1] A. Vershinin, Biological functions of carotenoids—Diversity and evolution, BioFactors 10 (1999) 99–104.
[2] L. Tao, A. Schenzle, J.M. Odom, Q. Cheng, Novel carotenoid oxidase involved in biosynthesis of 4,4′-diapolycopene dialdehyde, Appl. Environ. Microbiol. 71 (2005) 3294–3301.
[3] M. Albrecht, S. Takaichi, N. Misawa, G. Schnurr, P. Böger, G. Sandmann, Synthesis of atypical cyclic and acyclic hydroxy carotenoids in *Escherichia coli* transformants, J. Biotechnol. 58 (1997) 177–185.
[4] M. Albrecht, S. Takaichi, S. Steiger, Z.Y. Wang, G. Sandmann, Novel hydroxycarotenoids with improved antioxidative properties produced by gene combination in *Escherichia coli*, Nat. Biotechnol. 18 (2000) 843–846.

[5] G. Sandmann, M. Albrecht, G. Schnurr, O. Knorzer, P. Boger, The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*, Trends Biotechnol. 17 (1999) 233–237.
[6] G. Sandmann, Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures, ChemBioChem 3 (2002) 629–635.
[7] S. Steiger, S. Takaichi, G. Sandmann, Heterologous production of two unusual acyclic carotenoids, 1,1′-dihydroxy-3,4-didehydrolycopene and 1-hydroxy-3,4,3′,4′-tetradehydrolycopene by combination of the crtC and crtD genes from *Rhodobacter* and *Rubrivivax*, J. Biotechnol. 97 (2002) 51–58.
[8] P.C. Lee, A.Z.R. Momen, B.N. Mijts, C. Schmidt-Dannert, Biosynthesis of structurally novel carotenoids in *Escherichia coli*, Chem. Biol. 10 (2003) 453–462.
[9] C. Schmidt-Dannert, Engineering novel carotenoids in microorganisms, Curr. Opin. Biotechnol. 11 (2000) 255–261.
[10] C. Schmidt-Dannert, D. Umeno, F.H. Arnold, Molecular breeding of carotenoid biosynthetic pathways, Nat. Biotechnol. 18 (2000) 750–753.
[11] B.N. Mijts, P.C. Lee, C. Schmidt-Dannert, Identification of a carotenoid oxygenase synthesizing acyclic xanthophylls: combinatorial biosynthesis and directed evolution, Chem. Biol. 12 (2005) 453–460.
[12] D. Umeno, A.V. Tobias, F.H. Arnold, Evolution of the C_{30} carotenoid synthase CrtM for function in a C_{40} pathway, J. Bacteriol. 184 (2002) 6690–6699.
[13] D. Umeno, F.H. Arnold, A C_{35} carotenoid biosynthetic pathway, Appl. Environ. Microbiol. 69 (2003) 3573–3579.
[14] D. Umeno, F.H. Arnold, Evolution of a pathway to novel long-chain carotenoids, J. Bacteriol. 186 (2004) 1531–1536.
[15] D. Umeno, A.V. Tobias, F.H. Arnold, Diversifying carotenoid biosynthetic pathways by directed evolution, Microbiol. Mol. Biol. Rev. 69 (2005) 51–78.
[16] S. Ohnuma, K. Narita, T. Nakazawa, C. Ishida, Y. Takeuchi, C. Ohto, T. Nishino, A role of the amino acid residue located on the fifth position before the first aspartate-rich motif of farnesyl diphosphate synthase on determination of the final product, J. Biol. Chem. 271 (1996) 30748–30754.
[17] B. Wieland, C. Feil, E. Gloria-Maercker, G. Thumm, M. Lechner, J.M. Bravo, K. Poralla, F. Götz, Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4′-diaponeurosporene of *Staphylococcus aureus*, J. Bacteriol. 176 (1994) 7719–7726.
[18] N. Misawa, M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, K. Harashima, Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*, J. Bacteriol. 172 (1990) 6704–6712.
[19] D. Umeno, K. Hiraga, F.H. Arnold, Method to protect a targeted amino acid residue during random mutagenesis, Nucleic Acids Res. 31 (2003) e91.
[20] G. Britton, UV/visible spectroscopy, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Spectroscopy, vol. 1B, Birkhäuser Verlag, Basel, 1995, pp. 13–62.
[21] C.H. Eugster, Chemical derivatization: microscale tests for the presence of common functional groups in carotenoids, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Isolation and Analysis, vol. 1A, Birkhäuser Verlag, Basel, 1995, pp. 71–80.
[22] K. Schiedt, S. Liaaen-Jensen, Isolation and analysis, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Isolation and Analysis, vol. 1A, Birkhäuser Verlag, Basel, 1995, pp. 81–108.
[23] J.B. Davis, L.M. Jackman, P.T. Siddons, B.C.L. Weedon, Carotenoids and related compounds: Part XV. The structure and synthesis of phytoene, phytofluene, zeta-carotene, and neurosporene, J. Chem. Soc., C (1966) 2154–2165.
[24] B.H. Davies, C.J. Hallett, R.A. London, A.F. Rees, The nature of "theta-carotene", Phytochemistry 13 (1974) 1209–1217.
[25] S. Takaichi, Characterization of carotenes in a combination of a C_{18} HPLC column with isocratic elution and absorption spectra with a photodiode-array detector, Photosynth. Res. 65 (2000) 93–99.
[26] A. Hausmann, G. Sandmann, A single five-step desaturase is involved in the carotenoid biosynthesis pathway to beta-carotene and torulene in *Neurospora crassa*, Fungal Genet. Biol. 30 (2000) 147–153.
[27] S. Liaaen-Jensen, Combined approach: identification and structure elucidation of carotenoids, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Spectroscopy, vol. 1B, Birkhäuser Verlag, Basel, 1995, pp. 343–354.
[28] G. Englert, NMR spectroscopy, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Spectroscopy, vol. 1B, Birkhäuser Verlag, Basel, 1995, pp. 147–260.
[29] M. Albrecht, G. Sandmann, D. Musker, G. Britton, Identification of epoxy- and hydroxyphytoene from norflurazon-treated *Scenedesmus*, J. Agric. Food Chem. 39 (1991) 566–569.
[30] C.M. Aragón, F.J. Murillo, M.D. de la Guardia, E. Cerdá-Olmedo, An enzyme complex for the dehydrogenation of phytoene in *Phycomyces*, Eur. J. Biochem. 63 (1976) 71–75.
[31] R. Candau, E.R. Bejarano, E. Cerdá-Olmedo, In vivo channeling of substrates in an enzyme aggregate for beta-carotene biosynthesis, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 4936–4940.
[32] M.D. de la Guardia, C.M. Aragón, F.J. Murillo, E. Cerdá-Olmedo, A carotenogenic enzyme aggregate in *Phycomyces*: evidence from quantitative complementation, Proc. Natl. Acad. Sci. U. S. A. 68 (1971) 2012–2015.
[33] F.J. Murillo, S. Torres-Martinez, C.M. Aragón, E. Cerdá-Olmedo, Substrate transfer in carotene biosynthesis in *Phycomyces*, Eur. J. Biochem. 119 (1981) 511–516.
[34] G. Britton, Overview of carotenoid biosynthesis, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Biosynthesis and Metabolism, vol. 3, Birkhäuser Verlag, Basel, 1998, pp. 13–147.
[35] P.D. Fraser, N. Misawa, H. Linden, S. Yamano, K. Kobayashi, G. Sandmann, Expression in *Escherichia coli*, purification, and reactivation of the recombinant *Erwinia uredovora* phytoene desaturase, J. Biol. Chem. 267 (1992) 19891–19895.
[36] H. Linden, N. Misawa, D. Chamovitz, I. Pecker, J. Hirschberg, G. Sandmann, Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenes, Z. Naturforsch., C 46 (1991) 1045–1051.
[37] C.W. Wang, J.C. Liao, Alteration of product specificity of *Rhodobacter sphaeroides* phytoene desaturase by directed evolution, J. Biol. Chem. 276 (2001) 41161–41164.
[38] W.R. Farmer, J.C. Liao, Improving lycopene production in *Escherichia coli* by engineering metabolic control, Nat. Biotechnol. 18 (2000) 533–537.
[39] P. Krubasik, M. Kobayashi, G. Sandmann, Expression and functional analysis of a gene cluster involved in the synthesis of decaprenoxanthin reveals the mechanisms for C_{50} carotenoid formation, Eur. J. Biochem. 268 (2001) 3702–3708.
[40] A. Raisig, G. Sandmann, Functional properties of diapophytoene and related desaturases of C_{30} and C_{40} carotenoid biosynthetic pathways, Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1533 (2001) 164–170.
[41] R. Edge, D.J. McGarvey, T.G. Truscott, The carotenoids as anti-oxidants—A review, J. Photochem. Photobiol., B 41 (1997) 189–200.
[42] A.A. Woodall, S.W.M. Lee, R.J. Weesie, M.J. Jackson, G. Britton, Oxidation of carotenoids by free radicals: relationship between structure and reactivity, Biochim. Biophys. Acta Gen. Subj. 1336 (1997) 33–42.
[43] A.J. Young, G.M. Lowe, Antioxidant and prooxidant properties of carotenoids, Arch. Biochem. Biophys. 385 (2001) 20–27.
[44] J.D. Bu'Lock, The Biosynthesis of Natural Products, McGraw Hill, London, 1965.
[45] C.D. Reeves, The enzymology of combinatorial biosynthesis, Crit. Rev. Biotechnol. 23 (2003) 95–147.
[46] W. Schwab, Metabolome diversity: too few genes, too many metabolites? Phytochemistry 62 (2003) 837–849.
[47] C.L. Steele, J. Crock, J. Bohlmann, R. Croteau, Sesquiterpene synthases from grand fir (*Abies grandis*)—Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization and bacterial expression of delta-selinene synthase and gamma-humulene synthase, J. Biol. Chem. 273 (1998) 2078–2089.

[48] C.G. Jones, R.D. Firn, On the evolution of plant secondary chemical diversity, Philos. Trans. R. Soc. Lond., Ser. B Biol. Sci. 333 (1991) 273–280.
[49] R.D. Firn, C.G. Jones, An explanation of secondary product "redundancy", in: J.T. Romeo, J.A. Saunders, P. Barbosa (Eds.), Phytochemical Diversity and Redundancy in Ecological Interactions, vol. 30, Plenum Press, New York, 1996, pp. 295–312.
[50] R.D. Firn, C.G. Jones, The evolution of secondary metabolism—A unifying model, Mol. Microbiol. 37 (2000) 989–994.
[51] R.D. Firn, C.G. Jones, Natural products—A simple model to explain chemical diversity, Nat. Prod. Rep. 20 (2003) 382–391.
[52] S.I. Ohnuma, T. Nakazawa, H. Hemmi, A.M. Hallberg, T. Koyama, K. Ogura, T. Nishino, Conversion from farnesyl diphosphate synthase to geranylgeranyl diphosphate synthase by random chemical mutagenesis, J. Biol. Chem. 271 (1996) 10087–10095.
[53] A. Aharoni, L. Gaidukov, O. Khersonsky, Q.G.S. Mc, C. Roodveldt, D.S. Tawfik, The 'evolvability' of promiscuous protein functions, Nat. Genet. 37 (2005) 73–76.
[54] R. Colodner, Extended-spectrum beta-lactamases: a challenge for clinical microbiologists and infection control specialists, Am. J. Infect. Control 33 (2005) 104–107.