A highly selective biosynthetic pathway to non-natural C$_{50}$ carotenoids assembled from moderately selective enzymes

Maiko Furubayashi$^{1,1}$, Mayu Ikezumi$^1$, Shinichi Takaichi$^2$, Takashi Maoka$^3$, Hisashi Hemmi$^4$, Takuya Ogawa$^4$, Kyoichi Saito$^1$, Alexander V. Tobias$^5$ & Daisuke Umeno$^{1,6}$

Synthetic biology aspires to construct natural and non-natural pathways to useful compounds. However, pathways that rely on multiple promiscuous enzymes may branch, which might preclude selective production of the target compound. Here, we describe the assembly of a six-enzyme pathway in *Escherichia coli* for the synthesis of C$_{50}$-astaxanthin, a non-natural purple carotenoid. We show that by judicious matching of engineered size-selectivity variants of the first two enzymes in the pathway, farnesyl diphosphate synthase (FDS) and carotenoid synthase (CrtM), branching and the production of non-target compounds can be suppressed, enriching the proportion of C$_{50}$ backbones produced. We then further extend the C$_{50}$ pathway using evolved or wild-type downstream enzymes. Despite not containing any substrate- or product-specific enzymes, the resulting pathway detectably produces only C$_{50}$ carotenoids, including $\sim$90% C$_{50}$-astaxanthin. Using this approach, highly selective pathways can be engineered without developing absolutely specific enzymes.
One of the goals of synthetic biology is the engineering of organisms to synthesize useful chemicals, some of which may not be produced in nature\(^1\). Recent studies have begun to explore the space of novel metabolites by mixing, matching and laboratory-evolving biosynthetic genes from diverse sources in bacteria and yeasts\(^2\)–\(^8\). In extending natural pathways in novel directions, pathway engineers usually rely on the promiscuous activities (the ability to accept alternative substrates and/or synthesize alternative products) of recruited or mutated enzymes\(^4\)–\(^10\). Unfortunately, the use of such promiscuous enzymes, especially in succession, often generates pathway branch points to numerous byproducts, exponentially attenuating metabolic flux to the target molecule as pathway length is increased\(^9\)–\(^11\). Consequently, the chemical space we can practically access biosynthetically has been limited to a very small number of steps from natural structures.

Examination of biosynthetic pathways in nature has revealed a common strategy for achieving pathway selectivity: for many natural product pathways, the first committed enzymes serve as highly specific ‘pathway gatekeepers’\(^1\)\(^2\)\(^3\)\(^4\). With this type of pathway organization, enzymes further downstream in the pathway need only be moderately selective, since structurally similar but undesirable substrates will have been excluded by the upstream gatekeeper enzyme. Regrettably, engineering highly specific enzymes in the laboratory—in particular, elimination of activity on the native substrate or product—has proven difficult and time-consuming\(^14\)–\(^17\). Accordingly, constructing efficient and selective multi-step pathways to non-native metabolites is an extremely challenging problem. Approaches taken to improve pathway flux toward non-native pathway products include enzyme overexpression, deletion of competing endogenous enzymes\(^7\)\(^,\)\(^10\)\(^,\)\(^18\), as well as enzyme fusion, co-localization\(^11\), compartmentalization\(^19\) or attachment to scaffolds\(^20\). These strategies can redirect metabolic flux but do not prevent broad-specificity enzymes from creating unwanted branches. Here, we tackled the construction and focusing of flux for a pathway composed of six successive broad-specificity enzymes, in which an additional strategy was required. A novel strategy, which we term ‘metabolic filtering’, allowed us to implement a synthetic pathway to C\(_{50}\)-astaxanthin, a non-native, purple analogue of the carotenoid astaxanthin.

Carotenoid biosynthesis has provided an excellent model system for studying the biosynthetic discovery of new chemical structures\(^2\)\(^,\)\(^12\)\(^,\)\(^21\)\(^,\)\(^22\). The pigmentation of carotenoids facilitates screening for altered enzyme function and forms the basis for their nutritional and technological value. All >750 naturally occurring carotenoids are biosynthesized through the modification of C\(_{40}\) or C\(_{30}\) backbones. While the carotenoid backbone synthase CrtB or CrtM serves as the specific gatekeeper in bacterial C\(_{40}\) or C\(_{30}\) carotenoid pathways\(^2\)\(^,\)\(^3\)\(^,\)\(^8\)\(^,\)\(^23\), downstream enzymes are ‘locally specific’\(^12\)\(^,\)\(^21\), thereby allowing for the creation of non-native C\(_{40}\) or C\(_{30}\) carotenoids by mixing and matching (‘combinatorial biosynthesis’)\(^22\)\(^,\)\(^24\) and sometimes mutating\(^21\) the enzymes therein.

Previously, chemists synthesized 50-carbon analogues of β-carotene\(^25\) and astaxanthin\(^26\), C\(_{50}\)-β-carotene (\(9\)) and C\(_{50}\)-astaxanthin (\(12\); also called decapreno-β-carotene\(^25\) or decaprenoastaxanthin\(^26\); see Supplementary Note 1 for nomenclature rules). Compared with their natural C\(_{40}\) counterparts, the C\(_{50}\) carotenoids possessed novel purple (versus orange-to-red) pigmentation\(^25\) and superior antioxidant activity\(^27\). Given astaxanthin’s (6) importance (2010 sales of US$226 million\(^28\)) and extended biosynthetic pathway, we settled on C\(_{50}\)-astaxanthin (12) as a target non-native purple carotenoid product for this study. Almost a decade ago, some of us successfully constructed a non-native pathway to C\(_{50}\)-lycopene (8) in \(E.\) coli by engineering carotenoid enzymes\(^9\)\(^,\)\(^29\). However, the predominance of smaller carotenoid backbones and the low activity of carotenoid desaturase on the C\(_{30}\) backbone (7) limited production of C\(_{50}\)-lycopene (8) to only trace quantities (~a few % of total carotenoids). To further extend the C\(_{50}\) pathway to C\(_{50}\)-astaxanthin (12), it would be necessary to dramatically improve the selectivity of the nascent synthetic C\(_{50}\) pathway.

Herein, we describe our systematic efforts to rapidly establish a pathway that selectively produces C\(_{50}\)-astaxanthin (12) in \(E.\) coli. This time, we generated a collection of variants of the first two pathway enzymes, farnesyl diphosphate synthase (FDS) and carotenoid backbone synthase (CrtM), with slightly shifted size-range selectivities to identify the best pairings for the much more selective production of C\(_{50}\) backbone (7), via the ‘metabolic filtering’ effect (detailed below). This selective pathway to the C\(_{50}\) backbone allowed us to further evolve the downstream carotenoid desaturase CrtI for improved activity on C\(_{50}\) backbone. Subsequently, we co-expressed three broad specificity wild-type carotenoid modification enzymes, resulting in a six-enzyme pathway to C\(_{50}\)-astaxanthin (12) comprising 15 chemical transformation steps. Our synthetic pathway features minimal production of non-target carotenoid byproducts despite containing not a single enzyme with absolute specificity for its primary substrate or product.

Results

Design of a pathway to C\(_{50}\)-astaxanthin. To introduce a pathway to natural (C\(_{40}\)) astaxanthin (6) (Fig. 1) into \(E.\) coli\(^3\)\(^0\), geranylgeranyl diphosphate (C\(_{25}\)PP) synthase (CrtE) must be expressed to convert endogenous farnesyl diphosphate (C\(_{30}\)PP) into C\(_{25}\)PP, the precursor for C\(_{40}\) carotenoid synthesis. Phytene synthase (CrtB) catalyses the head-to-head condensation of two C\(_{20}\)PPs to phytene (1), the C\(_{40}\) carotenoid backbone. Phytene desaturase (CrtI) extends the double bond conjugation of C\(_{40}\) backbone, yielding lycopene (2). Terminal β-cyclization (catalysed by CrtY), hydroxylation (by CrtZ) and ketolation (by CrtW) then convert lycopene (2) into astaxanthin (6) (Fig. 2).

As with most secondary-metabolic enzymes, carotenoid-modifying enzymes, including those for β-cyclization, hydroxylation and ketolation (iv, v, vi), are known to have broad substrate specificity and bind to (or chemically modify) only a portion of their substrate (for example, the terminus)\(^3\)\(^1\)–\(^3\)\(^3\). Also, one of us found that Pantoaea anaanatis CrtI (iii) possessed weak but detectable desaturation activity on the C\(_{50}\) backbone\(^3\). In contrast, the two upstream enzymes responsible for carotenoid backbone synthesis, isoprenyl diphosphate synthase (i) and carotenoid backbone synthase (ii), are much more selective for their native product and substrate, respectively. However, through enzyme engineering, a broadened-specificity mutant of Geobacillus stearothermophilus farnesyl diphosphate (C\(_{15}\)PP) synthase (FDS) capable of C\(_{25}\)PP-synthesis, FDS\(_{Y894A}\) (i) was generated\(^3\)\(^4\), as was a set of broadened-specificity mutants of CrtM (a C\(_{30}\) carotenoid backbone synthase from Staphylococcus aureus) capable of C\(_{50}\) backbone synthesis from C\(_{25}\)PP, including CrtM\(_{f26A,W38A}\) (ii).

We designed a heterologous biosynthetic route to C\(_{50}\)-astaxanthin (12) by mimicking the astaxanthin (6) pathway (Fig. 1). Beginning with endogenous C\(_{30}\)PP, the pathway proceeds through (i) a two-step prenyl elongation to create geranylgeranyl diphosphate (C\(_{25}\)PP); (ii) head-to-head condensation of two C\(_{25}\)PPs to form the C\(_{50}\) backbone (7); (iii) six desaturation steps to generate the conjugated chromophore; and terminal (iv) β-cyclization; (v) hydroxylation; and (vi) ketolation (Fig. 1, Supplementary Fig. 1 and Supplementary Note 1).

With these two major-biological enzymes, carotenoid-modifying enzymes, including those for β-cyclization, hydroxylation and ketolation (iv, v, vi), are known to have broad substrate specificity and bind to (or chemically modify) only a portion of their substrate (for example, the terminus)\(^3\)\(^1\)–\(^3\)\(^3\). Also, one of us found that Pantoaea anaanatis CrtI (iii) possessed weak but detectable desaturation activity on the C\(_{50}\) backbone\(^3\). In contrast, the two upstream enzymes responsible for carotenoid backbone synthesis, isoprenyl diphosphate synthase (i) and carotenoid backbone synthase (ii), are much more selective for their native product and substrate, respectively. However, through enzyme engineering, a broadened-specificity mutant of Geobacillus stearothermophilus farnesyl diphosphate (C\(_{15}\)PP) synthase (FDS) capable of C\(_{25}\)PP-synthesis, FDS\(_{Y894A}\) (i) was generated\(^3\)\(^4\), as was a set of broadened-specificity mutants of CrtM (a C\(_{30}\) carotenoid backbone synthase from Staphylococcus aureus) capable of C\(_{50}\) backbone synthesis from C\(_{25}\)PP, including CrtM\(_{f26A,W38A}\) (ii).
Unfortunately, simple co-expression of the above genes (i, ii, iii, iv, v, vi) in E. coli resulted in the accumulation of a slew of non-target (mainly C40) carotenoids and C50-astaxanthin (12) was neither separated nor conclusively detected (Fig. 2b). Calculations reveal that based on the catalytic functions reported for the six enzymes, at least 642 different carotenoids can theoretically be biosynthesized from their co-expression (Supplementary Note 2, Supplementary Table 1 and Supplementary Fig. 2). This initial failure to synthesize C50-astaxanthin demonstrates that merely co-expressing the enzymes encoding all of the biochemical functions required in theory to synthesize a product does not guarantee a functional multi-step pathway in reality. Because the selectivities of the assembled pathway enzymes were not properly coordinated, carbon flux was diverted at key steps of the pathway along one or more undesirable paths. Convinced that enormous time and effort would be required to evolve the appropriate selectivities of the assembled pathway enzymes were not properly coordinated, carbon flux was diverted at key steps of the pathway along one or more undesirable paths. We instead attempted to search for FDS and backbone synthase (CrtM) variants that could combine to effect the selective production of C50 backbone.

Directed evolution of FDS for improved C25PP supply. Production of the C50 carotenoid backbone (7) requires the precursor C25PP. A handful of natural C25PP-producing enzymes have been described in the literature5,6, but they exhibited insufficient activity and selectivity for C25PP36. Instead, we had been expressing a variant of FDS (DFSY81A34) to produce C25PP. However, C25PP also accumulated and was readily incorporated into C30 and C45 backbones by engineered CrtM variants29 (Fig. 2a,c). These byproducts diverted carbon flux from the C50 pathway and outcompeted C50 backbone as substrates for the subsequent steps9,29.

Lacking a direct high-throughput screen for the production of C25PP, we subjected DFSY81A to random mutagenesis and instead screened the library for improved C20PP consumption (Fig. 3a). A library of random point mutants of DFSY81A was created, and was transformed into E. coli cells harbouring a lycopene-producing plasmid (pAC-crtE-crtB-crtI-idi), which were then spread on agar plates to form colonies. The rationale was that FDS variants with improved activity for converting C20PP into C25PP would more fully deplete the lycopene precursor C20PP, resulting in whiter colonies. Analysis of the three positive clones identified two additional substrate size-shifting mutations to FDS: T121A and V157A resulting in whiter colonies. Analysis of the three positive clones identified two additional substrate size-shifting mutations to FDS: T121A and V157A, which were spread on agar plates to form colonies.

Directed evolution of CrtM for improved C50 activity. Previously, some of the authors had engineered mutants of CrtM (a C30 backbone synthase) that could synthesize a C50 backbone (7), but only as a minor fraction of a complex mixture with C35 – C45 carotenoid backbones9,29 (Fig. 2a,c). We desired a better C50 backbone (7) synthase. The lack of a visual or colorimetric screen for identification of variants with improved activity for C50 backbone synthesis motivated us to conduct an indirect search for such variants: we searched for CrtM variants with approximately undiminished in vivo C30 synthase activity but reduced C50 synthase activity, both of which could be assayed by colony colour (Fig. 3c). Our hypothesis was that some of the resultant variants would possess an overall shifted preference for larger substrates (Supplementary Note 4 and Supplementary Fig. 5).

We chose CrtM38A, a broad-specificity variant that catalyses both C30 (C15PP + C13PP) and C50 (C20PP + C20PP) backbone synthesis29, as the parent for this evolution experiment.

**Figure 1 | Design of a C50-astaxanthin biosynthetic pathway in E. coli.** Non-natural steps are indicated with red arrows. Starting from endogenous C15PP, the pathway includes 15 biochemical steps catalysed by six enzymes (see Supplementary Fig. 1 to see all pathway intermediates). Bottom insets: colonies and cell pellets of E. coli expressing pathways to astaxanthin (left) and C50-astaxanthin (right) constructed in this study. DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; OPP, diphosphate unit.
(see Supplementary Note 4 for rationale). A library of genes encoding variants of CrtM_W38A was co-transformed with pAC-crtE-crtI-idi (see left panel in Fig. 3c) into E. coli cells and screened for maintenance of C30 activity for five rounds. Next, the resultant plasmid mixture was screened for diminished C30 synthase activity (right panel in Fig. 3c). Three mutants conferring the desired white phenotype were isolated. Analysis identified F233S as a new size-shifting substitution in CrtM (Supplementary Note 4 and Supplementary Table 3).

When F233S was combined with the previously identified size-altering substitutions 29 F26A or W38A, the resultant CrtM variants showed increased C50 synthase activity when co-expressed with FDS_Y81A, while still possessing C30 synthase activity (Fig. 3d and Supplementary Fig. 5). These variants also acquired unprecedented C55 synthase activity when co-expressed with a previously reported C25PP synthase mutant FDS_Y81A, FDS_Y81A, T121A, V157A. Similarly, CrtM F26A and CrtM F26A,W38A selectively produced C35 backbone when paired with FDS_Y81A, while still possessing C30 synthase activity when co-expressed with other CrtM variants (second and third row of Fig. 4a). Though no FDS variant is itself specific for a particular isoprenyl diphosphate product, a high degree of carotenoid backbone selectivity was achieved with several co-expressed CrtM variants.

**Matching FDS and CrtM variants for C50 metabolic filtering.**

Next, we investigated all eight combinations of the three FDS mutations (Y81A, T121A, V157A) as well as all eight combinations of the three CrtM mutations (F26A, W38A, F233S) by conducting an 8 x 8 in vivo combinatorial expression experiment (Fig. 4) in E. coli. The 64 carotenoid distributions were determined by HPLC.

This breeding experiment generated a diverse array of product distributions (Fig. 4a). Although none of the eight FDS or eight CrtM variants possesses stringent substrate or product selectivity, several pairings led to the production of a dominant carotenoid backbone. For example, CrtM_F26A,F233S can produce carotenoid backbones from C30 to C35, depending on the isoprenyl diphosphate synthase with which it is paired (seventh column of Fig. 4a). However, this variant produced C50 backbone with high proportion (>80% of total carotenoids) when paired with FDS_Y81A,T121A,V157A. Similarly, CrtM_F26A and CrtM_F26A,W38A selectively produced C35 backbone when paired with FDS_Y81A, T121A, V157A, while still possessing C30 synthase activity when co-expressed with other CrtM variants (second and third row of Fig. 4a). Though no FDS variant is itself specific for a particular isoprenyl diphosphate product, a high degree of carotenoid backbone selectivity was achieved with several co-expressed CrtM variants.

**Figure 2 | Co-expression of broad-specificity enzymes results in the complex mixture of undesired carotenoid byproducts.** (a) HPLC trace showing the backbone distribution resulting from co-expression of two enzyme variants, FDS_Y81A and CrtM_F26A,W38A, previously reported to have C25PP synthase activity and C50 backbone synthase activity, respectively. See Fig. 4 for the pathways generated by these enzymes. (b) Co-expression of the six broad-specificity enzymes (FDS_Y81A, CrtM_F26A,W38A, CrtI, CrtY, CrtW, CrtZ) encoding all of the biochemical functions required in theory to synthesize C50-astaxanthin results in a complex mixture of undesired carotenoids. (Supplementary Note 4 and Supplementary Table 3).
The presence of mutant FDS was subjected to random mutagenesis and screening for improved C20PP consumption. The library was visually assayed in colonies co-expressing the cellular activity of CrtM mutants. Carotenoid backbones produced by confounded with improved or altered activity on C40 backbone selectivity (pAC-C50 backbone (C50 backbone). Previously, FDS-CrtM variant pairs that produces C50 backbone with high transform them into E. coli (C50-selective pathway was a prerequisite for a simple visual screen of CrtI for C50 backbone (C50-lycopene (C50-lycopene (C50). Screening for C40 synthase activity in vivo pathway resulted in selective accumulation of C50 pigments without detectable accumulation of non-C50 carotenoids. We therefore attribute the low observed level of C50-lycopene (C50-lycopene) to instability, rapid decomposition or modification of the molecule. All of the improved C50 desaturases we isolated retained uncompromised C30 synthase function in the presence of CrtE and CrtI, followed by selection for reduction of C30 synthase function in the presence of crtn. Directed evolution of improved C50 backbone synthases. Random mutants of CrtMW38A were screened for maintenance of C40 synthase function in the presence of CrtM and CrtI, followed by selection for reduction of C30 synthase function in the presence of crtn. (d) The effect of selectivity-altering mutations on the cellular activity of CrtM mutants. Carotenoid backbones produced by E. coli harbouring pUC-crtM mutants and pAC-fdsY81A. FDSY81A provides C15PP, C20PP and C25PP for the CrtM variants. DNR, diaponeurosporene (C30 carotenoid); LYC, lycopene (C40 carotenoid).

**Figure 3** Directed evolution of C25PP synthase and C50 backbone synthase. (a) Directed evolution of a more efficient C25PP synthase. The Y81A mutant of FDS was subjected to random mutagenesis and screening for improved C20PP consumption. The library was visually assayed in colonies co-expressing crte, crtb and crtI; hits were colonies with reduced red (lycopene) pigmentation. (b) In vitro activity of FDS variants provided with DMAPP and [1-14C]PP as substrates. Note that the products have been dephosphorylated to the corresponding alcohol. See Supplementary Note 3 for details. (c) Directed evolution of improved C50 backbone synthases. Random mutants of CrtMW38A were screened for maintenance of C40 synthase function in the presence of CrtE and CrtI, followed by selection for reduction of C30 synthase function in the presence of crtn. We created a library of genes encoding CrtI variants and transformed them into E. coli cells harbouring one of the FDS-CrtM variant pairs that produces C50 backbone with high selectivity (pAC-fdsY81A,V157A-crtMF26A,W38A,F233S, Fig. 6a). Out of ~2,000 colonies surveyed, we isolated 6 with an intense red hue. Sequencing, followed by site-saturation mutagenesis identified top-performing variant CrtI_{N304P} as an efficient six-step C50 desaturase (Supplementary Note 7 and Supplementary Table 5). Expression of this variant in the context of a C50-selective pathway resulted in ~90% conversion of C50 backbones and some accumulation of the six-step desaturation product C50-lycopene (Fig. 6b). Interestingly, we observed far less C50-lycopene (Fig. 6b) than expected given the consumption of C50 backbone (7) and the accumulation level of C50-β-carotene (9) when a carotenoid cyclase was co-expressed (see below). We therefore attribute the low observed level of C50-lycopene (C50-lycopene) to instability, rapid decomposition or modification of the molecule. All of the improved C50 desaturases we isolated retained uncompromised C40 desaturase activity (Supplementary Fig. 8) and are therefore broadened-specificity enzymes, although CrtI_{N304P} did not appear to desaturate asymmetric C40 (C15PP + C25PP) carotenoid backbones. Because we had discovered FDS-CrtM pairings that yielded C50 backbones with high selectivity, co-expression of C50-β-carotene (9) essentially as the sole backbone.

**Construction of a selective C50-astaxanthin pathway.** We added downstream modifying enzymes to the resultant pathway for C50-lycopene (8). This molecule proved a substrate for the wild-type, broad-specificity lycopene cyclase (CrtY) from P. ananatis (31,37), such that co-expression of CrtY resulted in the accumulation of C50-β-carotene (9), essentially as the sole

**Directed evolution of CrtI for improved C50 activity.** Previously, P. ananatis CrtI was shown to have low but detectable C50 backbone (7) desaturation activity. Predictably, addition of CrtI to a C50-selective pathway yielded only a trace amount of C50-lycopene (8, Fig. 6b). A highly selective C50 backbone pathway was a prerequisite for a simple visual screen of CrtI for improved in vivo C50 desaturase activity that would not be confounded with improved or altered activity on C40 backbone (1), its native substrate (Fig. 6a and Supplementary Fig. 8a).

We created a library of genes encoding CrtI variants and transformed them into E. coli cells harbouring one of the FDS-CrtM variant pairs that produces C50 backbone with high selectivity (pAC-fdsY81A,V157A-crtMF26A,W38A,F233S, Fig. 6a). Out of ~2,000 colonies surveyed, we isolated 6 with an intense red hue. Sequencing, followed by site-saturation mutagenesis identified top-performing variant CrtI_{N304P} as an efficient six-step C50 desaturase (Supplementary Note 7 and Supplementary Table 5). Expression of this variant in the context of a C50-selective pathway resulted in ~90% conversion of C50 backbones and some accumulation of the six-step desaturation product C50-lycopene (8, Fig. 6b). Interestingly, we observed far less C50-lycopene (8) than expected given the consumption of C50 backbone (7) and the accumulation level of C50-β-carotene (9) when a carotenoid cyclase was co-expressed (see below). We therefore attribute the low observed level of C50-lycopene (8) to instability, rapid decomposition or modification of the molecule. All of the improved C50 desaturases we isolated retained uncompromised C40 desaturase activity (Supplementary Fig. 8) and are therefore broadened-specificity enzymes, although CrtI_{N304P} did not appear to desaturate asymmetric C40 (C15PP + C25PP) carotenoid backbones. Because we had discovered FDS-CrtM pairings that yielded C50 backbones with high selectivity, co-expression of C50-β-carotene (9) essentially as the sole backbone.

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carotenoid product (Fig. 7a). β-Rings are known to photochemically stabilize carotenoid chromophores27, and this may explain why the level of C50-β-carotene (9) was much higher than observed above for C50-lycopene (8) (Fig. 6b). Further addition of wild-type β-carotene ketolase CrtW and/or hydroxylase CrtZ from Brevundimonas sp. SD212, both known to be rather unspecific C40 carotenoid enzymes32,33, yielded the selective production of C50-zeaxanthin (10), C50-canthaxanthin (11) and C50-astaxanthin (12) (Fig. 7b). Thus, we readily extended the C50-lycopene (8) pathway to a novel C50-astaxanthin (12) pathway by simple co-expression of three

Figure 4 | Combinatorial co-expression of enzyme variants for selective production of carotenoid backbones. (a) Size distributions of carotenoids produced by combinatorial pairing of FDS and CrtM variants. Bars with stars represent asymmetric C40 backbones, produced from C15PP þ C25PP9. (b) Illustration of metabolic filtering. A modest shift in the precursor distribution effected by the choice of FDS variant, combined with a modest shift in backbone synthase substrate selectivity driven by the choice of CrtM variant can result in substantial focusing of pathway flux to a target product (for example, C35 or C50 carotenoids). Thicker arrows represent preferred enzyme selectivities, while larger dots represent greater metabolite concentrations. The boxes in a labelled i, ii and iii, correspond to the labelled diagrams in b. See Supplementary Note 6 and Supplementary Fig. 7 for a more extensive and quantitative illustration.

Figure 5 | Selective production of C30, C35, C40 and C50 backbones by selected combinations of FDS and CrtM variants. Peaks labelled with asterisks correspond to unidentified non-carotenoid compounds. Percentages refer to the mole fraction of total carotenoid backbones.

Figure 6 | Directed evolution of a C50 carotenoid desaturase. (a) CrtI was subjected to PCR mutagenesis and colonies were screened for increased pigmentation in cells predominantly producing the C50 backbone. Abolition of phytoene was prerequisite for assaying desired C50 (versus undesired C40) function. (b) C50 desaturase activity of CrtI and CrtIN304P in E. coli expressing FDSY81A,V157A and CrtMF26A,W38A,F233S. Insets show the colony hue of each transformant. CrtM*, CrtMF26A,W38A,F233S; FDS*, FDSY81A,V157A.
broad specificity wild-type downstream enzymes. C50-astaxanthin represented 90% of total carotenoids in the acetone extract; the other 10% was C50 backbone. No carotenoids with other backbones were detected. In a further application of metabolic filtering, selective pathways for the natural C40 counterpart carotenoids β-carotene (3), zeaxanthin (4), canthaxanthin (5) and astaxanthin (6) were also constructed (Fig. 7) simply by matching FDSY81A,T121A,V157A and CrtMF26A,F233S or C40 (FDST121A,V157A and CrtMF26A,W38A) backbones were additionally transformed with the indicated genes to produce (a) C50-β-carotene or C40 (natural) β-carotene, (b) C50 oxo-carotenoids or C40 (natural) counterparts. Shown from left to right are the pathway constructs, cell pellets, HPLC chromatograms, absorption spectra and the corresponding carotenoid structure. As illustrated, the plasmid constructs and expression contexts were identical for all wild-type and variants of each gene under comparison (see Supplementary Table 10).

Discussion

The promiscuity of many biosynthetic enzymes enables the creation of novel pathways to non-natural metabolites12,38,39. In our case and in others9,10,24, however, the use of broad-specificity enzymes, especially in the early steps of a novel pathway, has led to a low-titre mixture of numerous products. In cases where a heterologous pathway must compete with an endogenous pathway for substrate, or where ‘leakage’ of pathway intermediates to or from endogenous enzymes is an issue, solutions such as enzyme overexpression, deletion, fusion, scaffolding or sequestration have shown effectiveness7,10,18–20. However, these approaches cannot help with the problem of promiscuity-driven pathway branching within the heterologous pathway itself, because the undesirable intermediates both originate from and are processed by heterologous pathway enzymes. Alterations to the selectivity of one or more heterologous pathway enzymes are therefore required to improve flux and selectivity for the desired novel metabolite.

A perfectly ‘lossless’ synthetic pathway might require a highly specific enzyme at every step, but we expected it would be immensely difficult to convert FDS and CrtM into variants with exquisite selectivity for their preferred activities14–17,40. Instead, we coordinated two successive moderately selective pathway enzymes to ‘share’ the role of pathway gatekeeper12,13 by matching them such that the first enzyme’s product range ‘overlaps’ partially but appropriately with the second enzyme’s substrate range (see Supplementary Note 6). In this way, the two enzymes act together as a ‘metabolic filter’ to channel flux along
the preferred path. Although some off-pathway (non-carotenoid) byproducts may be generated, C₅₀ backbone (7) titre was greatly improved from an initial state (Fig. 2a) of ~40 μg gDCW⁻¹ to ~770 μg gDCW⁻¹ (Fig. 5). Crucially, the reduction of on-pathway (mainly C₅₀ carotenoid) byproducts enabled the directed evolution of a C₅₀ carotenoid desaturase (Fig. 6) and subsequent extension of the C₅₀ pathway by 12 additional transformations to C₅₀-astaxanthin (12). Thus, metabolic filtering was the key that brought the C₅₀ pathway to prominence and allowed us to elevate C₅₀-astaxanthin (12) from undetectable (Fig. 2) to a level comparable to that of C₅₀ astaxanthin produced in E. coli in this and other studies (Supplementary Fig. 9 and Supplementary Table 6).

This work demonstrates that selective multi-enzyme pathways can be constructed through the judicious matching of individually broad-specificity enzymes and proves that exquisitely specific gatekeeper enzymes are not necessary for achieving a highly selective pathway. This is an encouraging result, with the potential to impact the design and implementation of other synthetic metabolic pathways. In the near future, as synthetic biologists conceive and attempt to establish pathways that diverge ever farther from the natural and use ever-increasing numbers of laboratory-evolved enzymes, the problem of undesired pathway branching due to heterologous enzyme promiscuity will become more widespread and the need for solutions such as this one will grow more critical.

We believe our results and analysis provide a framework for understanding how complex and precise biosynthetic networks can be built out of imperfect biosynthetic parts, either by synthetic biologists or natural evolution. Since tremendous evolutionary effort is usually required for an enzyme to acquire stringent substrate or product specificity, it makes sense that alternative evolutionary routes to pathway selectivity would have been discovered by nature. Recently, Nam et al. showed that 37% of all E. coli enzymes are promiscuous and that such enzymes are responsible for 65% of the bacterium’s known metabolic steps. Nature therefore seems frequently content to mitigate rather than eliminate the catalytic promiscuity of its enzymes. Our work tangibly demonstrates an additional facet of this phenomenon, and, because so few genetic changes (mutation and lateral gene transfer) were required for us to exploit metabolic filtering, it is very likely that nature discovered this strategy long ago. Mitigation rather than elimination of enzyme promiscuity would preserve nature’s ability to rapidly discover new metabolites, and could thus provide some organisms with a selective advantage. Similarly, the ‘patchwork hypothesis’ posits that the selective metabolic pathways we observe in nature evolved from ‘leaky’ assemblies of broad-specificity enzymes. Though individual enzyme specialization has generally been accepted as the primary driver of this evolution, the ‘distributed specificity’ strategy described herein appears to be a more evolutionarily parsimonious and perhaps, more likely mechanism.

Methods

Strains and reagents

E. coli XL10-Gold (Tet<sup>+</sup> Amr<sup>+</sup> lcr) A(mcrCB-lcdSMR- mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hf [P proAB lacP2.1M15 Tn10 (Tet<sup>+</sup> Amy Cam<sup>–</sup>)] (Stratagene. La Jolla, CA) was used for cloning, while XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [P proAB lacP2.1M15 Tn10 (Tet<sup>+</sup>)] (Stratagene) was used for carotenoid production experiments. Cells were grown in Luria-Bertani (LB) in Lennox media for cloning or preculture and in Terrific Broth (TB) media for carotenoid production. Carbencilin (50 μg ml⁻¹; Sigma-Aldrich, St Louis, MO) and/or chloramphenicol (30 μg ml⁻¹; Nacalai Tesque, Kyoto, Japan) were supplemented where appropriate. For protein overexpression and purification, BL21-AI (Life Technologies, Carlsbad, CA) was used. For induction, 0.2% (w/v) L-阿拉伯糖 (Nacalai Tesque) and 0.1 mM IPTG (Nacalai Tesque) was added as an inducer.

Plasmid construction.

Genes and plasmids used in this paper are listed in Supplementary Table 7 and Supplementary Table 8, respectively. Plasmid maps are shown with Supplementary Fig. 11. Plasmids with the prefix ‘pUC’ are based on the pUC18m vector, which has an EcoRI-XhoI-A Apal multi-cloning site under a lac promoter. Plasmids with the prefix ‘pAC’ are based on the pACm vector. The plasmids for the downstream enzymes (crt, crtY, crtW and crtZ) and their derivatives are based on the pUCm vector. The vector was made by replacing the lac promoter of pUC18m with that of pUCm. The pBADHisA vector (Invitrogen), together with multi-cloning site-encoding plasmids (EcoRI-XhoI-A Apal-Spel-Clal-HindIII) placed immediately downstream the araBAD promoter. The detailed constructions for each plasmid are provided in Supplementary Note 8. Primer sequences are listed in Supplementary Table 9.

Plasmid combinations in each experiment

Plasmid combinations used in each experiment are listed in Supplementary Table 10. In brief, for directed evolution and analysis of CrtM, FDS and CrtI (Figs 3 and 6, Supplementary Figs 3 and 5d,e), the gene to be mutated was in the pUC18m vector, and additional genes co-expressed for screening were in the pACm vector. For the production of more than 10 ml of 0.9% (w/v) NaCl, and then repeated by centrifugation. The cultures were shaken at 37°C for 16h. The cultures were then diluted 100-fold into 1 ml of fresh TB medium in 250 ml flasks, which were shaken at 30°C (200 r.p.m.) for 48h.

Culture conditions for carotenoid backbones

Plasmids harbouring fds or crtM variants were transformed into XL1-Blue cells, which were then plated on LB-agar plates and incubated at 37°C for 24h. Colonies were inoculated into 2 ml of LB medium in culture tubes, which were shaken at 37°C for 16h. The cultures were then diluted 100-fold into 1 ml of fresh TB medium in 250 ml flasks, which were shaken at 30°C (200 r.p.m.) for 48h.

Culture conditions for desaturated carotenoids

For the desaturated carotenoids (cells transformed with fds variants, crtM variants and one or more downstream enzymes such as crtI variants, crtY, crtW and crtZ), the procedure was the same as that for carotenoid backbones, except for the culturing time: the cultures were shaken for 36h, followed by the addition of a-arabinose to a final concentration of 0.2% (w/v) and an additional 36 h of shaking, unless otherwise indicated.

Carotenoid extraction and HPLC analysis

Thirty millilitres of each cell culture were centrifuged at 3,300g for 4°C for 15 min. The cell pellets were washed with 10 ml of 0.9% (w/v) NaCl, and then repelleted by centrifugation. The cultures were extracted by adding 10 ml of acetone followed by vigorous shaking. One millilitre of hexane and 35 ml of 1% (w/v) NaCl were added, the samples were centrifuged at 3,300g for 15 min, and the carotenoid-containing hexane phase was collected. The hexane was then evaporated under a stream of N₂. The extracts were then dissolved in 100 μl of hexane or (1:1) methanol/THF for separation. A 25-μl aliquot of the final extract was analysed by a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector.

For the analysis of carotenoid backbones (co-expression culture of fds and crtM variants; Figs 2a, 3d, 4 and 5, Supplementary Figs 3g, 4 and 6), a Spherisorb ODS 2 column (250 mm x 4.6 mm, 5 μm, Waters, Milford, MA) was used. Carotenoids were eluted with mobile phase of acetonitrile/isopropanol (6:4 v/v, 1 ml min⁻¹) was performed.

For desaturated carotenoids (co-expression culture of fds, crtM and crtI variants, and/or crtY variants; Figs 6a and 7a, and Supplementary Fig. 8b–d), a Spherisorb ODS 2 column (see above) was used. The mobile phase was acetonitrile/tetrahydrofuran/methanol (30:7:63 v/v, 1.5 ml min⁻¹).

For the analysis of xanthophylls (carotenoids with oxygenic groups; co-expression culture of fds, crtM, crtI variants and crtY, together with crtW and/or crtz), we used two different elution conditions. For Fig. 2a,b, TSKgel ODS column and the following gradient elution were employed: solvent A (methanol/water 95:5) for 5 min, gradient from 100% solvent A to 100% solvent B (methanol/tetrahydrofuran 7:3) over the next 5 min, then 100% solvent B for an additional 10 min. For Fig. 7b, a MAbondapak column (100 × 8 mm, RCM-type, Waters) was used with methanol as the isocratic mobile phase (1 ml min⁻¹).

Individual carotenoids were quantified by their peak areas using a calibration curve generated with known amounts of β-carotene, together with the molar extinction coefficient (ε) of β-carotene (138,900 M⁻¹ cm⁻¹ at 450 nm (ref. 45) and dividing by the ε value for the carotenoid in question (summarized in Supplementary Table 11). Production weights of carotenoids were then normalized to the dry cell weight (DCW) of each culture. DCW was calculated using an OD<sub>max</sub> DCW calibration curve.

High-throughput analysis of carotenoid production

Analysis of carotenoid production using FDS variants (Supplementary Fig. 3a) or CrtM variants (Supplementary Fig. 5d,e) was performed as follows: transformants (harboursing plasmids indicated in Supplementary Table 10) were plated onto LB Lennox agar plates with appropriate antibiotic concentrations. The colonies were picked and inoculated into 500 μl LB Lennox media in a 96-well deep well plate and cultured at 37°C, 1,000 r.p.m. for 16h. An aliquot (40 μl) of these pre-cultures was
transferred to 2 ml TB in a 48-well deep well plate and cultured at 30 °C, 1,000 rpm for 48 h. Cells were collected, washed with saline, centrifuged to obtain cell pellets and the supernatants were discarded. After brief vortex, 1 ml acetone was added to each of the cell pellets, immediately followed by vortex for 1 min and then centrifugation. The absorbance spectra (350–650 nm at 5nm interval) of the resultant extracts were determined using a SpectraMax Plus 340 (Molecular Devices, CA). The pigmentation level of each culture was determined from the lambdaw (470 nm) of each sample. For FLM (fluorescence) of the resultant extract, using the molar absorption coefficients of diaponeurosporene and 488 nm (147,000 M⁻¹ cm⁻¹), and lycopene68 (185,000 M⁻¹ cm⁻¹).

**Pigment identification.** Identification of each carotenoid was based on its HPLC retention time and absorption spectrum in the mobile phase. After purification, relative molecular masses of the carotenoids were determined by field-desorption mass spectrometry (FD-MS) with an M-2,500 double-focusing gas chromatograph/mass spectrometer equipped with an FD apparatus (Hitachi, Tokyo, Japan). High-resolution molecular masses were determined by fast atom bombardment (FAB) MS with 3-nitrobenzyl alcohol as a matrix using a JMS-700 (JEOL, Tokyo, Japan). The 1H NMR (500 MHz) and/or 13C NMR (125 MHz) spectra of C50 carotenoids in CDCl3 were measured at room temperature with a Unity INOVA-500 system (Varian, Palo Alto, CA). Circular dichroism (CD) spectra were recorded in ether at room temperature with a JASCO-J 300 spectropolarimeter. For chemical shift assignments of NMR, see Supplementary Fig. 12. Since the carbon number of C50 carotenoids is larger than that of natural (C40) carotenoids, the IUPAC-IUB numbering rule of C40 carotenoids could not be applied to C50 carotenoids. We applied a new numbering rule to the C50 carotenoids as we indicate in Supplementary Fig. 12. C50-b-carotene (9). Peak 9 of the HPLC chromatogram in Fig. 7a was collected and analysed. 1H NMR (500 MHz, CDCl3): δ 6.65 (4H, d, J = 15, H11, H-15, 15′, 11′), 6.65 (2H, m, H-19, 19′), 6.28 (2H, d, J = 15, H15, H-16, 16′), 6.63 (2H, d, J = 15, H-12, 12′), 6.28 (2H, broad d, J = 11, H-11, H11, 11′), 6.15 (2H, d, J = 11, H-14, 14′), 6.15 (2H, d, J = 11, H-18, 18′), 6.15 (2H, d, J = 11, H-10, 10′), 6.10 (2H, d, J = 16, H-7, 7′), 4.00 (2H, m, H-3, 3′), 2.39 (2H, ddd, J = 17, 10 Hz, H-14, 4′), 2.05 (2H, d, J = 17, 10 Hz, H-14, 4′) (Supplementary Fig. 13). The absorbance spectra (350–650 nm at 5 nm interval) of the resultant extract were determined using a SpectraMax Plus 384 (Molecular Devices, CA) centrifugation. The absorbance spectra (350–650 nm at 5 nm interval) of the resultant extract were determined using a SpectraMax Plus 384 (Molecular Devices, CA).

In vitro reaction of FDS variants. The assay was performed as described previously34 with slight modifications. Each assay mixture contained, in a final volume of 200 μl, 1 μmol of MgCl2, 10 μmol of NH4Cl, 10 μmol of 2-mercaptoethanol, 10 μmol of Tris-HCl buffer (pH 8.5), an appropriate amount of purified FDS variant and [1-14C]IPP (3 Ci mol⁻¹) and DMAP as substrates (see Supplementary Fig. 4 for experimental conditions). The enzyme amount was set to a level such that <20% of the IPP or DMAP would be consumed under the following reaction conditions. The mixture was incubated at 30 °C for 5 min, and the reaction was stopped by adding 400 μl of ice-chilled saturated NaCl solution. The reaction mixture was then shaken with 1 ml of 1-butanol that had been saturated with NaCl. The radioactivity in the 1-butanol layer was determined with a liquid scintillation counter (LSC-5100, Aloka, Japan). The resulting polypropylene diphenylphosphates in 1-butanol were treated with acid phosphatase at 37 °C overnight according to the method of Fuji et al.19. The hydrolyzates were extracted with n-pentane and analysed by reversed-phase thin layer chromatography using a Silica gel plate, KLC-18F (GE Healthcare, USA) or RP18 (Merck, Germany), developed with acetone/H2O (9:1). The radioactivities of the spots were measured with a Typhoon-FLA 7000 (GE Healthcare, USA).

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**Protein purification.** E. coli BL21-AI (Life Technologies, Carlsbad, CA) was transformed with pET-fds_con, Fresh colonies were picked, pre-cultured for 14 h, and inoculated into 40 ml LB carbenicillin in 200 ml flasks. Each culture was shaken at 37 °C, 200 rpm and induced with 100 μM IPTG and 0.2% (w/v) arabinose when the OD600 reached 0.6–0.8 (approximately 1.5 h). Each culture was then shaken for an additional 4 h before harvest.

One milliliter of B-PER reagent (Pierce Biotechnology, Rockford, IL), supplemented with DNase I, lysozyme, 0.1 mM PMSF, 500 μM NaCl, and 25 mM imidazole, was used to lyse each cell pellet. FDS variants were purified from the lysate using a His SpinTrap column and desalted using a PD Miniclad G-10 column (both from GE Healthcare), following the manufacturer’s instructions. The buffers used were: binding buffer (50 mM Tris-HCl pH 8.5, 500 mM NaCl, 25 mM imidazole), elution buffer (50 mM Tris-HCl buffer pH 8.5, 500 mM NaCl, 500 mM imidazole) and desalting buffer (50 mM Tris-HCl pH 8.5). The concentrations of the resultant protein variants were measured by BCA assay (Pierce BCA Protein Assay Kit), and the purified proteins were stored at −20 °C after adding 5 mM DTT and 15% glycerol.
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Author contributions
M.F. and D.U. conceived the study and designed the experiments. M.F. performed the majority of the experiments, including pathway construction and directed evolution. M.I. conducted the directed evolution of FDS. S.T. and T.M. conducted the analysis and structural determination of the novel carotenoids. H.H. and T.O. performed the project. M.F., A.V.T. and D.U. analysed the data and wrote the manuscript.

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