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Research Article

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Modular Assembly of the Astaxanthin Biosynthetic Pathway via SNARE-Derived Coiled-Coil Domains

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Abstract

Spatial organization of metabolic enzymes into multi-enzyme complexes is a powerful strategy to enhance biosynthetic flux and product yield in microbial cell factories. However, the current repertoire of orthogonal protein-protein interaction modules available for synthetic enzyme assembly remains limited. Here, we explore SNARE-derived α -helical coiled-coil domains from *Saccharomyces cerevisiae* as a new class of programmable interaction modules for metabolic engineering. Multiple sequence alignment of yeast SNARE coiled-coil regions revealed strongly conserved heptad residues, and AlphaFold3 modelling predicted high interaction propensity (ipTM > 0.6) for selected t-SNARE/v-SNARE peptide pairs. The predicted interactions were validated *in vivo* in *E. coli* by confocal microscopy: fusion of Vam3, Vam7, or Vti1 t-SNARE peptides to eGFP and of the v-SNARE peptide Nyv1 to mCherry produced co-localized punctate foci, in stark contrast to the diffuse cytoplasmic distribution of the unfused fluorophores. We then applied this modular assembly strategy to the heterologous astaxanthin biosynthetic pathway by tethering the carotenoid β -hydroxylase CrtZ and the carotenoid ketolase CrtW through SNARE coiled-coil peptide pairs. The best-performing strain, Z-vam3-W-Nyv1, produced 1 mg/g DCW of astaxanthin, a 2-fold improvement over the unassembled parent strain (~ 0.5 mg/g DCW), without any detectable effect on host growth kinetics. Collectively, these results establish SNARE-derived coiled-coil peptides as a biocompatible, modular, and programmable toolkit for spatially organizing biosynthetic enzymes, with broad applicability to metabolic engineering and biomanufacturing.

Keywords: SNARE proteins; coiled-coil domain; modular enzyme assembly; substrate channeling; metabolic engineering; astaxanthin; *Escherichia coli*

1 Introduction

The rapidly evolving field of synthetic biology is fundamentally reshaping the principles of metabolic engineering. At the core of this transformation lies the rational design of intracellular spatial organization, which is critical for the efficient assembly and precise regulation of metabolic pathways.

Inspired by the cooperative behavior of natural enzyme complexes, modular assembly strategies have emerged as indispensable tools for enhancing target product formation while suppressing undesirable side reactions. By minimizing the diffusional loss of metabolic intermediates and directing metabolic flux more efficiently, these approaches markedly improve overall pathway performance (Dubey and Tripathi, 2021). This strategy is rooted in the recognition of the highly crowded intracellular milieu, in which macromolecules occupy 20–30% of the cytoplasmic volume and reduce molecular diffusion rates by three- to four-fold relative to dilute aqueous solutions (Ellis, 2001). Evolution has addressed this constraint through specialized structural adaptations; for example, the substrate tunnel in tryptophan synthase enables efficient intermediate transfer between the α - and β -active sites, thereby substantially enhancing catalytic efficiency (Hyde et al., 1988; Dunn et al., 2008; Raboni et al., 2009). Many metabolic enzymes naturally assemble into multi-enzyme complexes—exemplified by polyketide synthases—that enable direct substrate channeling between active sites (Mabesoone et al., 2024). This phenomenon, termed “metabolic channeling,” accelerates reaction rates, sequesters potentially toxic intermediates, and affords refined regulatory control (Kerfeld et al., 2018). Consequently, the spatial organization of enzymes through modular assembly has become a cornerstone strategy for enhancing biosynthetic output.

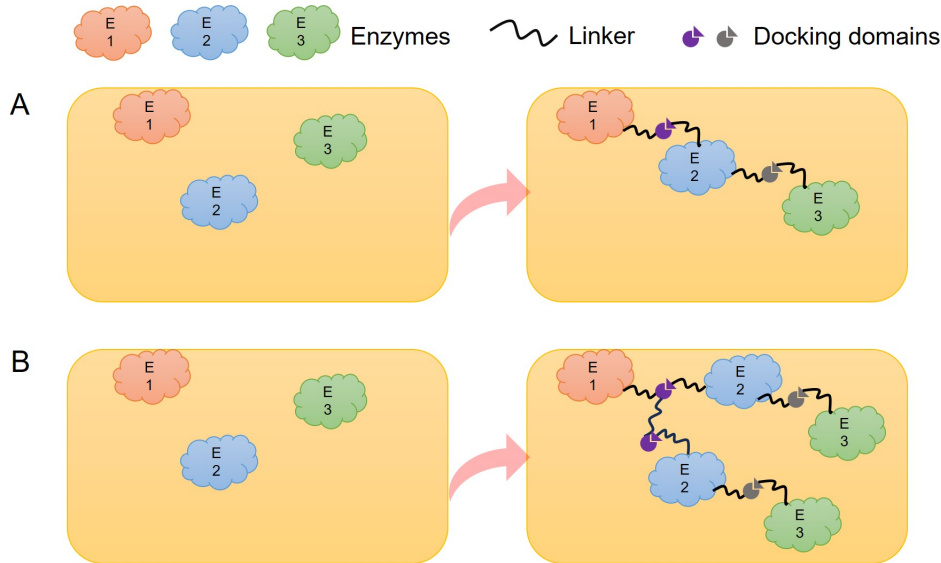


Fig. 1 Multi-enzyme assembly strategy utilizing protein interaction domains (A) Symmetric assembly: Single binding domain fused to each enzyme enables pairwise interaction. (B) Tunable assembly: Enzyme-specific fusion configurations (one or two binding domains) optimize catalytic efficiency based on individual enzyme kinetics. E1:Enzymes 1, E2:Enzymes 2, E3:Enzymes 3; Linker: a short peptide between the enzyme and the binding domain.

Current mainstream platforms for multi-enzyme organization can be broadly classified into three categories. (i) **Immobilized enzyme systems**, in which enzymes are anchored onto solid supports to achieve spatial proximity (Srere et al., 1973; Sheldon and van Pelt, 2013). (ii) **Biomimetic assembly systems**, which draw inspiration from natural supramolecular complexes; for instance,

the dockerin-cohesin modules found in cellulosomes allow modular reconfiguration of enzyme ensembles and have been shown to enhance cellulose degradation efficiency by up to 7.2-fold (Bayer et al., 2004; Artzi et al., 2017). (iii) **Fusion protein engineering**, in which bifunctional enzymes are generated through genetic fusion using peptide linkers, thereby enabling substrate channeling between covalently tethered active sites (Elleuche, 2015). Beyond these classical approaches, multi-domain protein scaffolds have been developed by concatenating specific protein-binding domains (Dueber et al., 2009). In particular, bio-orthogonal peptide pairs such as RIDD-RIAD and SpyTag-SpyCatcher have emerged as versatile and widely applicable tools for orchestrating multi-enzyme assembly (Hatlem et al., 2019; Berckman and Chen, 2020), and docking domains derived from modular polyketide synthases have likewise been repurposed to construct cascade-enzyme assemblies for natural-product biosynthesis (Sun et al., 2022). Nevertheless, the current repertoire of well-characterized interaction modules remains limited, restricting the broader application of modular assembly strategies across diverse biomanufacturing contexts.

SNARE proteins play an essential role in vesicular membrane trafficking by driving membrane fusion through the assembly of stable four-helix bundles—termed SNAREpins—that form via α -helical coiled-coil domains (Weber et al., 1998; Jahn and Scheller, 2006). These proteins are generally categorized as t-SNAREs, which reside on target membranes, or v-SNAREs, which localize to vesicle membranes. Despite their functional diversity, SNARE proteins exhibit highly conserved coiled-coil regions. A notable example is provided by the yeast SNAREs Sec9p and Sso1p, whose coiled-coil domains associate through precise “lock-and-key” interactions governed by conserved heptad repeats (Fasshauer et al., 1998; Burri and Lithgow, 2004). The binding free energy of these interactions ($\Delta G \approx -50$ kJ/mol) exceeds that of commonly used bio-orthogonal pairs such as SpyCatcher-SpyTag ($\Delta G \approx -40$ kJ/mol), thereby minimizing off-target assembly and mitigating potential metabolic disruption (Hatlem et al., 2019; Reddington and Howarth, 2015). In addition, SNAREpin formation is allosterically regulated by Sec1/Munc18 (SM) family chaperones, which act as templates to ensure rapid and accurate SNARE assembly (Südhof and Rothman, 2009; Zhang and Hughson, 2021). The dynamic properties of these assemblies can be further engineered—for example, by tuning helix length or introducing phosphorylation sites—to construct molecular switches responsive to cellular cues such as ATP/ADP ratios or pH. Moreover, the intrinsic kinetic barrier of SNAREpin formation (activation energy $E_a \approx 80$ kJ/mol) offers a tunable sensing mechanism for metabolic stress, underscoring the potential of SNARE-derived modules in the design of dynamically regulated synthetic systems (Li et al., 2016).

Astaxanthin exhibits exceptional antioxidant capacity—approximately 1,000-fold greater than that of vitamin E—and has therefore attracted broad interest for its health-promoting properties

(Higuera-Ciapara et al., 2006; Ambati et al., 2014). It has been recognized as a promising therapeutic candidate for the management of inflammation, cancer, and other chronic disorders (Fakhri et al., 2018). In light of its substantial commercial and biomedical value, recent advances in metabolic engineering have enabled the development of microbial platforms for the heterologous production of astaxanthin (Park et al., 2018; Zhang et al., 2018). In the present study, we employed coiled-coil domains derived from yeast SNARE proteins as modular linkers to spatially organize the astaxanthin biosynthetic pathway into a multi-enzyme complex. Although SNARE proteins are functionally diverse both across and within species, their coiled-coil interaction motifs are highly conserved at the sequence level. Importantly, these isolated domains retain their specific binding capacity when excised from the full-length protein, as confirmed by confocal microscopy. In engineered *Escherichia coli*, fusion of selected SNARE coiled-coil peptides to CrtZ and CrtW promoted enzyme co-localization *in vivo* and led to a substantial improvement in astaxanthin titer. Collectively, this modular assembly strategy provides a robust and programmable platform for constructing synthetic enzyme complexes with tailored functions, highlighting its broad applicability in metabolic engineering and biomanufacturing.

2 Materials and Methods

2.1 Materials and reagents

PCR amplification was carried out using Phanta Super-Fidelity DNA Polymerase (Vazyme, P501, Nanjing, China). All oligonucleotide primers were custom-synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Amplified DNA fragments were purified with the TIANgel Midi Purification Kit (TIANGEN BIOTECH, DP209, Beijing, China), and DNA assembly was performed using the ClonExpress MultiS One Step Cloning Kit (Vazyme, C113, Nanjing, China). Plasmid DNA was isolated using the TIANprep Mini Plasmid Kit (TIANGEN BIOTECH, DP107, Beijing, China). QuickCut restriction enzymes were obtained from TaKaRa (Beijing, China), and Luria–Bertani (LB) broth medium was supplied by Qidian Co., Ltd. (Ningbo, China). The astaxanthin standard was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). All organic solvents used for product extraction and HPLC analysis were obtained from Sigma–Aldrich (St. Louis, MO, USA), whereas inorganic salts were supplied by Qidian Co., Ltd. (Ningbo, China).

2.2 Strain construction and growth conditions

For plasmid assembly, the required DNA fragments were generated by PCR amplification using specific template–primer combinations and subsequently joined using the ClonExpress MultiS One Step Cloning Kit (Vazyme, C113, Nanjing, China). Routine cloning and plasmid maintenance were

performed in *E. coli* DH5 α cultivated on selective LB agar plates (1.5% w/v) or in liquid LB broth. Antibiotic selection was applied at the following final concentrations, depending on the construct: kanamycin (50 μ g/mL), ampicillin (100 μ g/mL), or chloramphenicol (25 μ g/mL). The integrity of all plasmid constructs was confirmed by restriction enzyme digestion followed by Sanger sequencing. Recombinant *E. coli* C43 strains harbouring the relevant expression plasmids were then generated for two complementary applications: heterologous astaxanthin biosynthesis and live-cell co-localization imaging. All plasmids, bacterial strains, and oligonucleotide primers used in this study are listed in Supplementary Data Files 1 and 2.

2.3 Fluorescence confocal microscopy

Confocal imaging was performed on a Leica STELLARIS 5 laser scanning confocal microscope (Leica Microsystems, Germany). Single colonies of strains expressing the fluorescent protein fusions were inoculated into 10 mL of LB broth supplemented with the appropriate antibiotics. Cultures were incubated at 30 °C with shaking at 200 rpm until reaching an optical density at 600 nm (OD₆₀₀) of 0.6–0.8. Protein expression was then induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4 h. Following induction, cells were harvested by centrifugation and visualized using an oil-immersion objective. Laser excitation intensities (488 nm for eGFP; 561 nm for mCherry) were carefully optimized to avoid cross-talk between fluorescence channels. Acquired images were processed and analysed with the Leica Application Suite X (LAS X) software platform.

2.4 Pigment extraction

Seed cultures were established by inoculating single colonies into 5 mL of LB broth containing the appropriate antibiotics and were incubated overnight at 37 °C with orbital shaking (200 rpm). For production-scale cultivation, a 1% (v/v) aliquot of seed culture was transferred to 50 mL of fresh, antibiotic-supplemented LB medium in 250 mL baffled flasks. Fermentation was performed at 30 °C with agitation at 200 rpm until the cultures reached mid-logarithmic phase (OD₆₀₀ = 0.6–0.8), at which point recombinant protein expression was induced with 0.1 mM IPTG. Samples were harvested 4 h after induction for subsequent analysis. All experiments included three biological replicates initiated from independent colonies. Cell biomass was collected by centrifugation (7,000 $\times g$, 5 min), and pigment extraction was performed by resuspending the pellets in an equal volume of acetone and incubating the suspension overnight in complete darkness until the biomass was fully decolourized.

2.5 High-performance liquid chromatography (HPLC)

Following extraction, the suspensions were clarified by centrifugation ($12,000 \times g$, 10 min), and the supernatants were filtered through $0.22 \mu\text{m}$ nylon membrane filters (ANPEL, Shanghai, China) prior to chromatographic analysis. Separation was performed on an Agilent 1260 HPLC system equipped with a reversed-phase C18 column (Agilent Technologies, Inc., CA, USA; $150 \text{ mm} \times 4.6 \text{ mm}$, $5 \mu\text{m}$ particle size). The column temperature was maintained at 25°C , and detection was carried out at 475 nm . A binary solvent gradient was applied at a constant flow rate of 1.0 mL/min , with mobile phase A consisting of water and mobile phase B consisting of methanol/isopropyl alcohol (8:2, v/v). The gradient programme was as follows: 0–5 min, 50–100% B (linear); 5–25 min, 100% B (isocratic); 25–30 min, 100–50% B (linear). Astaxanthin was quantified by external calibration with the authentic standard, applying appropriate dilution factors. Reported values represent the mean of three replicate injections, and final yields are expressed as milligrams of astaxanthin per gram of cell dry weight (mg/g DCW).

2.6 Growth curve measurements

Seed cultures of each strain were inoculated at a 1:100 dilution into liquid LB medium. Cultures of Z-W, Z-vam3-W-Nyv1, Z-vam7-W-Nyv1, and Z-Vti1-W-Nyv1 were supplemented with ampicillin to a final concentration of $100 \mu\text{g/mL}$ and incubated at 37°C with orbital shaking (200 rpm). Aliquots were withdrawn at defined intervals (0, 2, 4, 5, 6, 7, 8, 10, 12, and 24 h) for measurement of OD_{600} . Growth curves were generated by plotting the mean cell density ($\text{OD}_{600} \pm \text{SD}$) against cultivation time over the 24 h monitoring period.

3 Results

3.1 AlphaFold3 predicts interactions between SNARE-derived coiled-coil peptides

SNARE proteins constitute a functionally conserved family that plays an essential role in vesicular membrane trafficking. Their mechanism of action relies on the assembly of stable four-helix bundles—termed SNAREpins—through α -helical coiled-coil domains, thereby driving spatiotemporally precise membrane fusion. Based on their subcellular localization, SNAREs are classified as t-SNAREs (on the target membrane) or v-SNAREs (on the vesicle membrane)(Fig.2 A). To identify conserved sequence features within their coiled-coil regions, we performed a multiple sequence alignment (MSA) of the coiled-coil domains from all available yeast SNARE proteins, comprising 16 t-SNAREs and 5 v-SNAREs. The alignment revealed distinct patterns of residue conservation in each group: among the t-SNAREs, position 35 was strictly conserved as glutamine (Q) and position 60 was also highly

185 conserved, whereas among the v-SNAREs, conserved residues were observed at positions 20, 26, 35,
186 40, and 50(Fig.2 B and C). These evolutionarily conserved motifs provide a structural basis for the
187 rational design of orthogonal protein–protein interaction modules.

188 Building on these observations, we selected representative coiled-coil domain sequences from
189 several t-SNARE/v-SNARE pairs and predicted their interactions using AlphaFold3. The analysis
190 indicated a substantial predicted interaction propensity for all selected coiled-coil pairs, with interface
191 predicted Template Modeling (ipTM) scores consistently exceeding 0.6, supporting their potential to
192 form specific heterodimeric complexes.

193 **3.2 In vivo validation of predicted interactions in *E. coli* using fluorescent** 194 **fusion proteins**

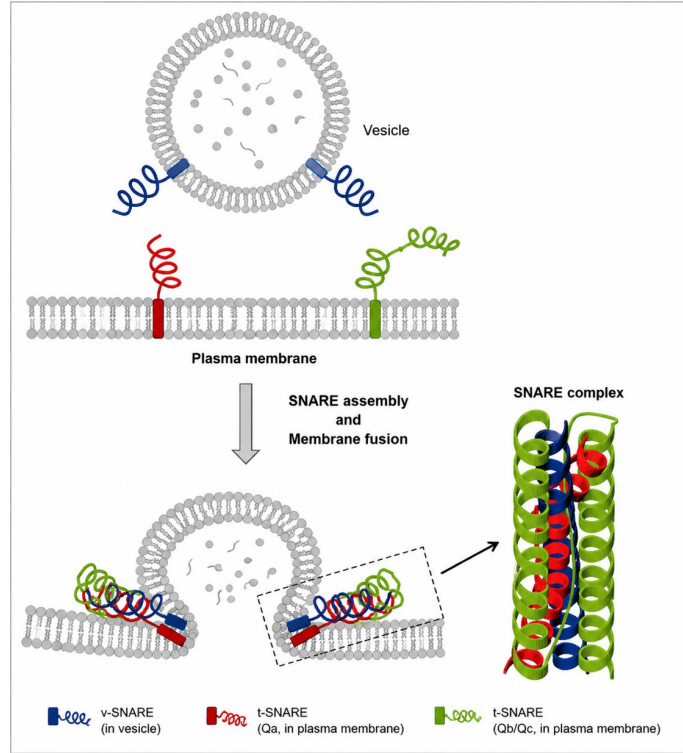
195 Guided by the AlphaFold3 predictions, three coiled-coil peptide pairs derived from yeast SNARE pro-
196 teins were selected to assess their interaction potential *in vivo* in *E. coli*. The peptides were taken from
197 the following domains: the t-SNARE peptides Vam3 (residues 190–252), Vam7 (residues 250–312),
198 and Vti1 (residues 124–186), and the v-SNARE peptide Nyv1 (residues 167–227). Each t-SNARE
199 peptide was genetically fused to eGFP—generating Vam3_{190–252}-eGFP, Vam7_{250–312}-eGFP, and
200 Vti1_{124–186}-eGFP—whereas the v-SNARE peptide Nyv1_{167–227} was fused to mCherry(Fig.3 A). Flu-
201 orescence co-localization was then analysed in *E. coli* cells co-expressing one of the following combi-
202 nations: Vam3_{190–252}-eGFP + Nyv1_{167–227}-mCherry; Vam7_{250–312}-eGFP + Nyv1_{167–227}-mCherry;
203 or Vti1_{124–186}-eGFP + Nyv1_{167–227}-mCherry.

204 In control cells expressing unfused eGFP and mCherry, both fluorescent signals were uniformly
205 distributed throughout the cytoplasm. In marked contrast, cells expressing the coiled-coil fusion
206 proteins displayed pronounced aggregation of fluorescence: eGFP fused to the t-SNARE peptides
207 formed discrete punctate structures, while mCherry-Nyv1 consistently accumulated as dense foci,
208 typically at the cell poles or in mid-cell regions(Fig.3 B). The clear transition from a diffuse to a
209 clustered distribution, together with the overlapping localization patterns of the two fluorophores,
210 provides direct visual evidence that the SNARE-derived coiled-coil peptides mediate specific protein–
211 protein interactions in living *E. coli* cells, thereby driving the assembly of defined multi-enzyme
212 complexes.

213 **3.3 Coiled-coil modular assembly enhances astaxanthin production**

214 Modular assembly strategies inspired by the synergistic organization of natural enzyme complexes
215 provide a powerful means to enhance target metabolite production while suppressing by-product
216 formation. By confining metabolic intermediates and directing metabolic flux, this approach can

A



B

	10	20	30	40	50	60	
SFT1 YEAST/7-69	7	SQTESNDRKLEGLANKLATFRNINQEI	GDRAVSDSSVINQMTDSLGSMTD	IKNSSSR	ITRS	69	
STX8 YEAST/164-202	164	QQQLLEQDSHLGALSQSIGRTHDISL	DLNNEIVSQND	SL	-----	202	
UFE1 YEAST/255-317	255	LNQKNEQLKKVETINKTILDIVNIQ	NELSNHLTVQSQNINLMNNQDD	IELNKKGNKEL	RKA	317	
SEC9 YEAST/588-650	588	DEMELEIDRNLDQIQVSNRLKKMAL	TTGKELDSQOKRLNNEESTDD	LDINLHMNTNRL	LAGI	650	
TLG2 YEAST/244-306	244	EAYLRERDEEITQLARGVLEVSTIF	REMQLDVLVDQGTIVDRIDYNLE	NTVVELKSADK	ELNKA	306	
TLG1 YEAST/132-194	132	EQMLREQDVHLDGTHKTMQNLHI	QAQTMGDELENQGLLDNMD	EGMDGVVKNLARG	RRLQLEWV	194	
BET1 YEAST/52-114	52	ASLESQSEEQMGAMGQRIKALKSL	SLKMGDEIRGSNQTIDQLGDT	TFHNTSVKLR	KRTFGNMEM	114	
SED5 YEAST/249-311	249	NVYLQERNRAVETIESTIQEVGNL	FQQLASMVQEGEVIQRIDAN	VDDIDLNISGA	QRELLKY	311	
VTI1 YEAST/124-186	124	HAILQKSGDRLKASRIANETEGIG	SQIMMDLRSORETLENARQ	TLFQADSYVD	KSITKTKM	186	
SPO20 YEAST/330-392	330	NQMEIDLGNLEQIKAVSGDLKIM	AHAFGREFEAQNTRMFDI	ENNVQQADN	ALQAKRYLEKV	392	
SEC9 YEAST/434-496	434	KFTKQSSVASTRNTLKMAQDAER	AGMNTLGMGLGHQSEQLNN	VEGNLDLMKVQ	NKVADEKVAEL	496	
VAM7 YEAST/250-312	250	MQMVRDQEQELVALHRIIQAGR	GLALEMNEELQTONELL	TALEDDVNT	GRRLQIANKKARHF	312	
VAM3 YEAST/190-252	190	TIHQERSQQIGRIHTAVQEVNAI	FHQLGSLVKEQGEQVTTID	ENISHLHDNM	QNAKQLTRA	252	
SSO1 YEAST/190-252	190	LAEVQARHQELKLEKSMAL	ETQLFNDMEELVIEQ	QENVVDIDKN	VEDAQDVEQGVGHTDKA	252	
SSO2 YEAST/194-256	194	LAEVQARHQELKLEKSMAL	ETQLFNDMEELVIEQ	QENVVDIDKN	VEDAQDVEQGVGHTDKA	256	
PEP12 YEAST/195-257	195	QNLIEQRDQEI	SNTERGITELNEVFKDLG	SVVQQQGLVDNIE	ANIYTTSDNTQLASDEL	RKA	257

C

	10	20	30	40	50	60	
YKT6 YEAST/140-200	140	AIMKVQQLDETKIVLHKT	ENVLQGEKLDNLVDK	SESTASSKMYKQ	KKNSSCC	IM	200
SEC22 YEAST/132-192	132	NLDQLNQLVGVKQIMSKN	IEDLLYRGDSLDKMS	DMSSSLKETS	KRYRKSQ	KINFDLLIS	192
SNC2 YEAST/27-87	27	KTAALRQEI	DDTVGIMRDNINK	VAERGERLTSIED	KADNLAI	SAQGFKRGANVR	KQMWYK
SNC1 YEAST/28-88	28	RTAELQAEI	DDTVGIMRDNINK	VAERGERLTSIED	KADNLAV	SAQGFKRGANVR	KAMMYK
NYV1 YEAST/167-227	167	IGDATEDQIK	DKVIQIMNDNIDK	FLERQERVSL	LVDKTSQ	LNSSSNKFRK	AVNIKEIMWQ

Fig. 2 Sequence conservation analysis of yeast SNARE coiled-coil domains. (A) SNARE complex assembly and membrane fusion mechanism. Schematic representation of SNAREpin formation between vesicle-associated SNARE (v-SNARE, blue) and target membrane-associated SNAREs (t-SNAREs, red and green), mediated by their coiled-coil domains (helical structures), facilitating membrane fusion. (B) Multiple sequence alignment of coiled-coil domains from 16 yeast t-SNAREs. (C) Multiple sequence alignment of coiled-coil domains from 5 yeast v-SNAREs. Residue conservation is visualized using a color gradient (purple: high conservation; white: low conservation). MSA was performed using Clustal Omega with default parameters. Key conserved residues discussed in the text (e.g., Gln³⁵ in t-SNAREs) are indicated.

substantially improve overall pathway efficiency. In recent years, engineering of the astaxanthin biosynthetic pathway in *E. coli* for enhanced yield has attracted considerable research interest.

In previous work, we constructed an astaxanthin-producing *E. coli* strain, designated Z-W, which served as the baseline strain in this study (Fig. 4). However, the astaxanthin titer of Z-W remained limited, reaching only approximately 0.5 mg/g DCW. To overcome this bottleneck, we implemented a spatial organization strategy based on the SNARE-derived coiled-coil peptide pairs identified above.

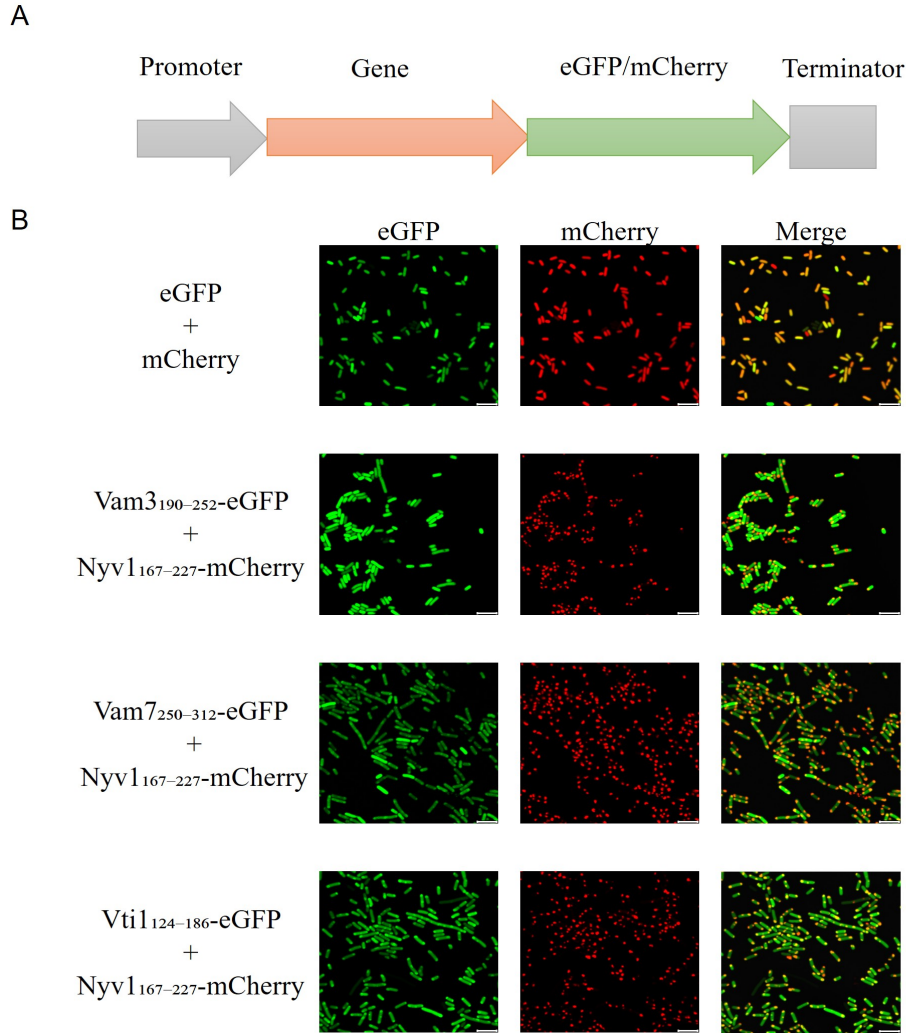


Fig. 3 Intracellular validation of coiled-coil interactions using fluorescent fusion proteins in *E. coli*. (A) Plasmid design strategy. Schematic of expression constructs featuring promoter (gray arrow), catalytic gene (orange arrow), fluorescent reporter gene (eGFP: green arrow; mCherry: green arrow), and terminator (gray rectangle). (B) Confocal microscopy analysis of protein co-localization. Left: eGFP fluorescence (488 nm excitation); Center: mCherry fluorescence (561 nm excitation); Right: Merged channels with DIC overlay (yellow indicates co-localization); Scale bars: 5 μ m. Fluorescence distribution patterns demonstrate: Top row (Control): Diffuse cytoplasmic localization of unfused eGFP and mCherry. Three Bottom rows (Coiled-coil fusions): Punctate co-localization (yellow foci) confirms complex formation mediated by engineered SNARE domains. Experiments were repeated at least three times with similar results.

Specifically, peptides from the t-SNAREs Vam3, Vam7, and Vti1 were fused via flexible linkers to the carotenoid β -hydroxylase CrtZ (UniProt: Q44262), while the v-SNARE peptide Nyv1 was fused to the carotenoid ketolase CrtW (UniProt: Q44261). This design exploits the specific binding affinity of the coiled-coil motifs to promote complex formation between CrtZ and CrtW, with the aim of establishing substrate channeling during astaxanthin biosynthesis and thereby increasing pathway flux and product yield.

Consistent with this hypothesis, strains expressing the coiled-coil-fused enzyme pairs (Z-vam3-W-Nyv1, Z-vam7-W-Nyv1, and Z-Vti1-W-Nyv1) exhibited markedly elevated astaxanthin production compared with the baseline. The most productive strain, Z-vam3-W-Nyv1, reached a yield of 1 mg/g DCW—a 2-fold improvement over the Z-W parent (Fig.5). These results indicate that the engineered coiled-coil peptides act as functional molecular linkers that promote close apposition of

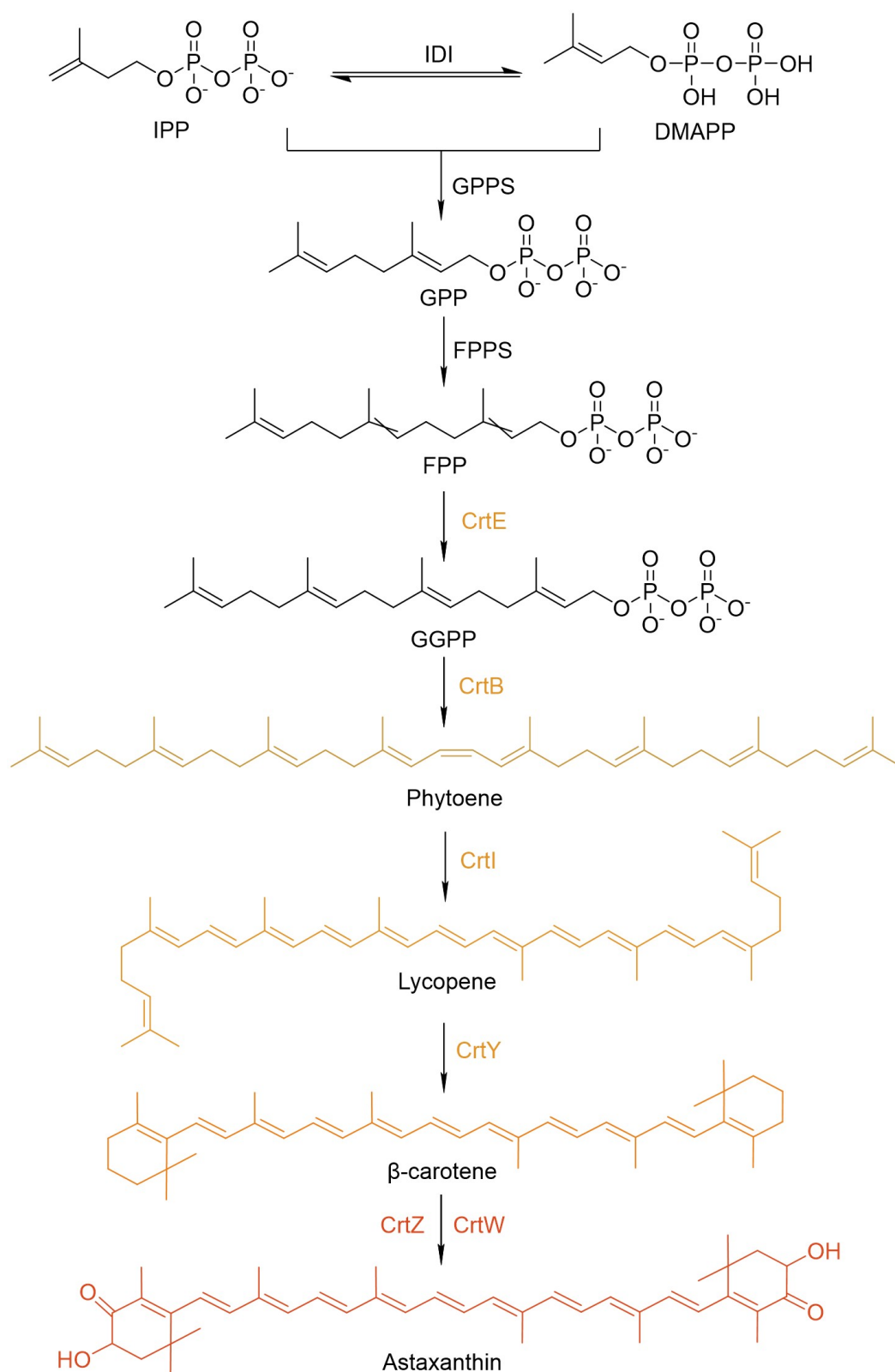


Fig. 4 Engineered astaxanthin biosynthetic pathway in *E. coli*. The black enzymes are endogenous host enzymes; the orange enzymes are heterologous enzymes from plasmid pACCAR16ΔcrtX (kindly provided by Prof. Wang Qiang); the red enzymes are engineered enzymes (CrtZ/CrtW) with coiled-coil interaction domains. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate. IdI, isopentenyl diphosphate isomerase; GPPS, geranylgeranyl diphosphate synthase; FPPS, farnesyl pyrophosphate synthase; CrtE, geranylgeranyl diphosphate synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase; CrtY, lycopene β-cyclase; CrtZ, β-carotene hydroxylase; CrtW, β-carotene ketolase.

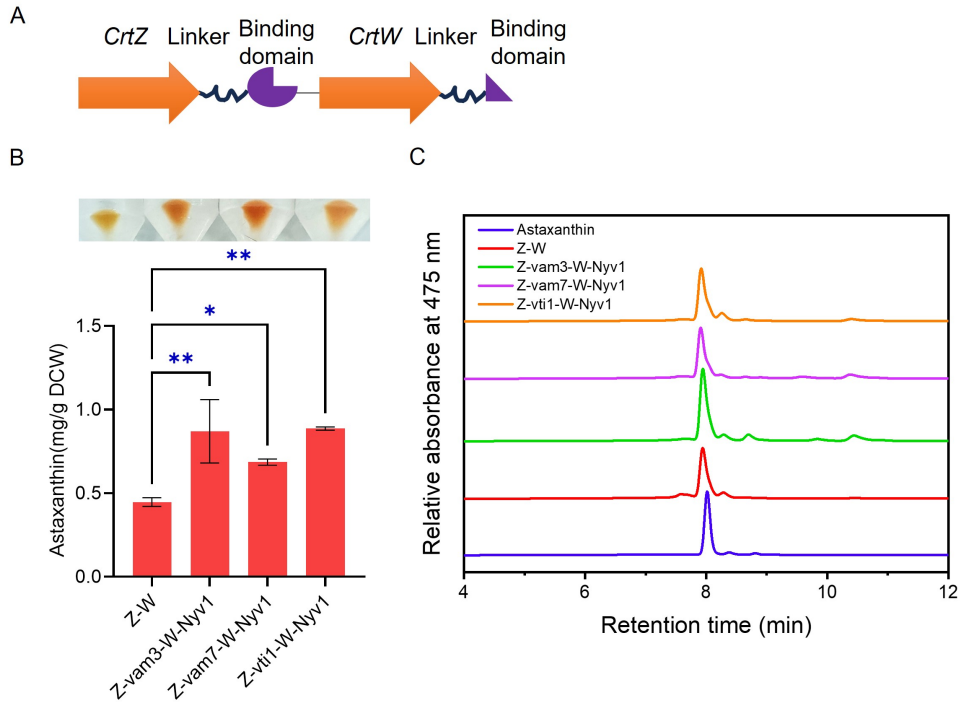


Fig. 5 Enhanced astaxanthin production through coiled-coil-mediated enzyme assembly. (A) Enzyme fusion strategy. Schematic of CrtZ/CrtW fusion constructs with engineered interaction domains. Catalytic domains (orange arrows) are connected via flexible linkers (gray curves) to orthogonal coiled-coil modules (purple model). (B) The astaxanthin production in different engineered strains. Z-W without coiled-coil domains as control; strains Z-vam3-W-Nyv1 with coiled-coil domains from Vam3 and Nyv1, respectively; Z-vam7-W-Nyv1 with coiled-coil domains from Vam7 and Nyv1, respectively; Z-Vti1-W-Nyv1 with coiled-coil domains from Vti1 and Nyv1, respectively; the above image of histogram is different strains of bacterial precipitation color. (C) HPLC quantification: Chromatograms showing astaxanthin production (monitored at 475 nm). Blue trace: purified astaxanthin standard (retention time: 8.0 min). Data represent mean $\pm$ SD (n=3 biological replicates). Statistical significance determined by one-way ANOVA with Duncan's test. (**P < 0.01).

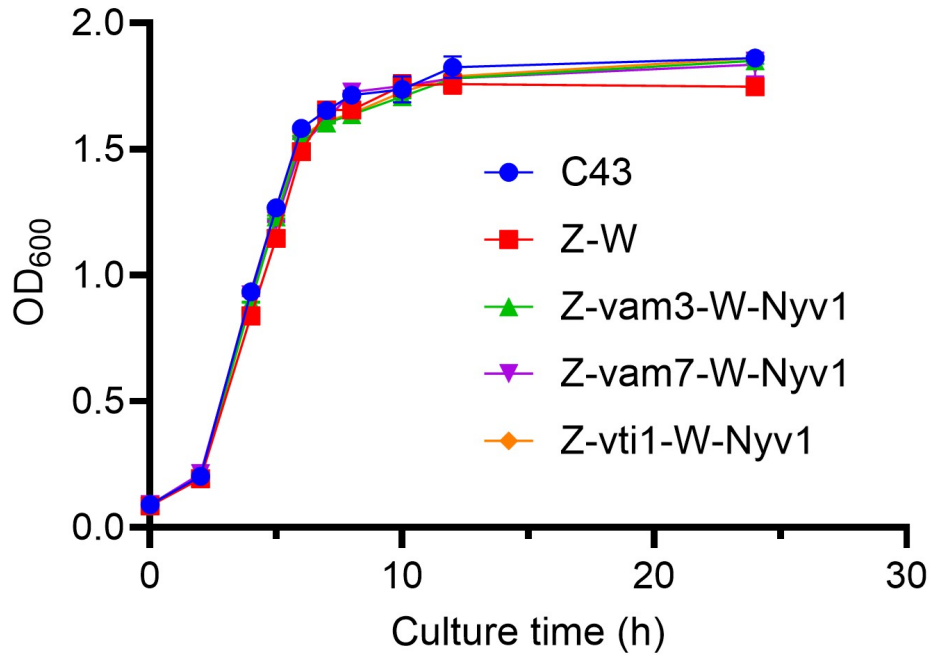


Fig. 6 Growth curves of different strains.

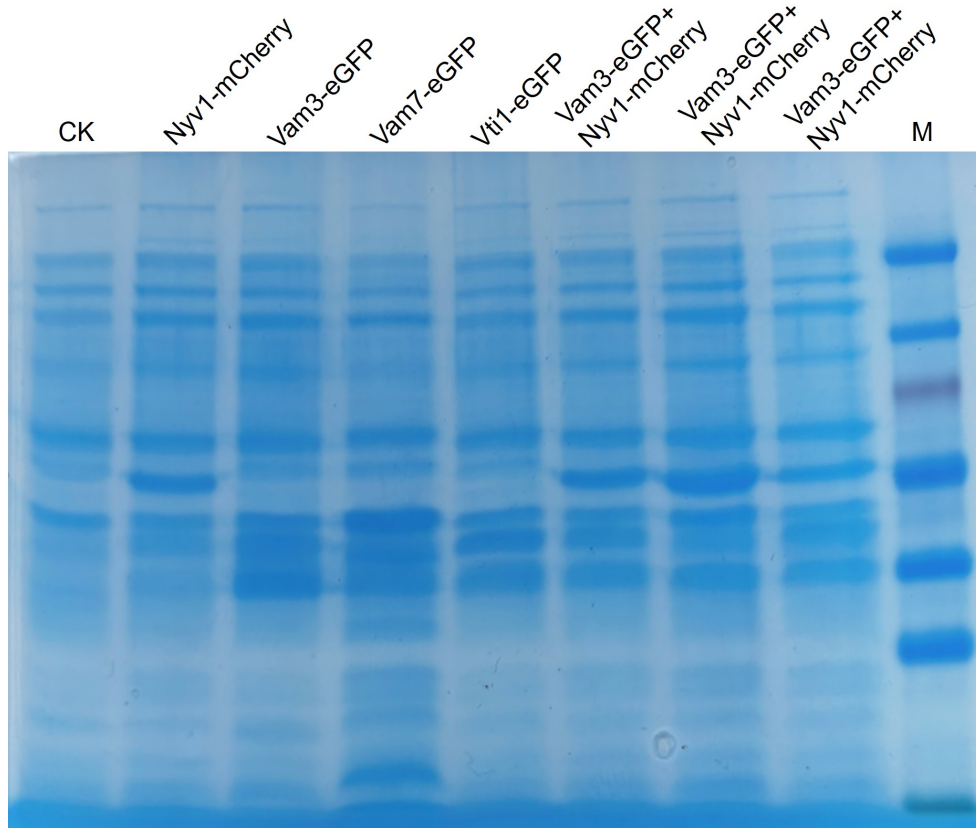


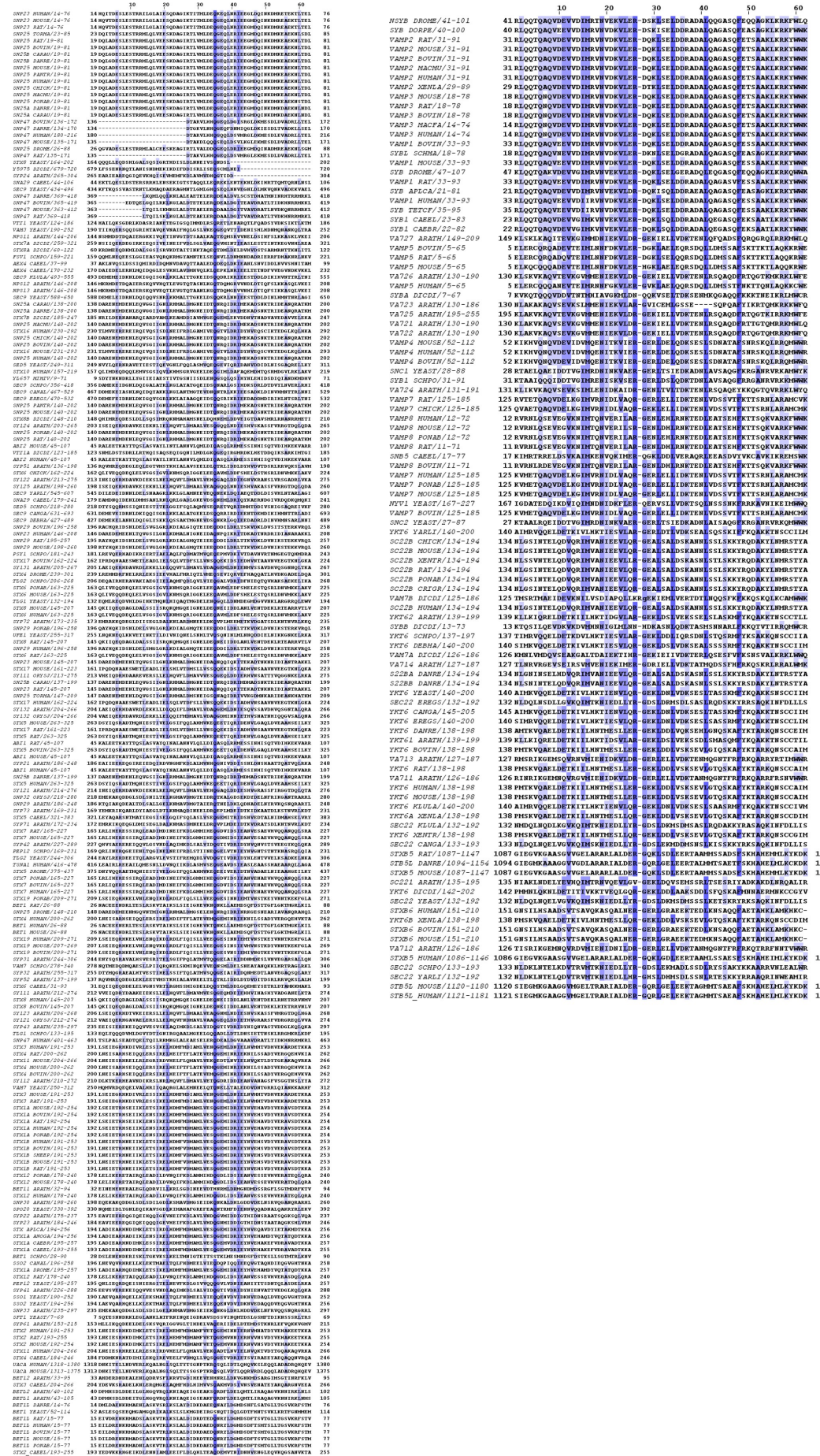
Fig. 7 The proteins expression of different strains. CK is C43 strains. M is protein marker. Vam3, Vam7, Vti1 and Nyv1 represent the coiled-coil domains from corresponding protein, respectively.

CrtZ and CrtW; the resulting spatial proximity is expected to enhance the transfer of catalytic intermediates between the two enzymes, leading to a substantial increase in astaxanthin biosynthesis efficiency.

3.4 SNARE-derived coiled-coil peptides do not impair *E. coli* growth

The coiled-coil peptides used in this study originate from *Saccharomyces cerevisiae* SNARE proteins, where they serve as essential structural elements of the vesicular trafficking machinery. As these heterologous sequences derive from a phylogenetically distant organism, we first assessed their potential cytotoxicity in the *E. coli* host and examined whether the recombinant fusion proteins (CrtZ-coiled-coil and CrtW-coiled-coil) would impair cellular growth.

To this end, we compared the growth kinetics over 24 h of the following strains: C43 (empty vector control), Z-W (baseline astaxanthin-producing strain), Z-vam3-W-Nyv1, Z-vam7-W-Nyv1, and Z-Vti1-W-Nyv1. Growth profiling revealed consistent physiological dynamics across all engineered strains, which entered logarithmic phase at approximately 2 h and reached stationary phase by approximately 6 h, with no discernible differences in the timing of growth-phase transitions. Quantitative analysis confirmed that strains expressing the coiled-coil fusion proteins displayed growth kinetics



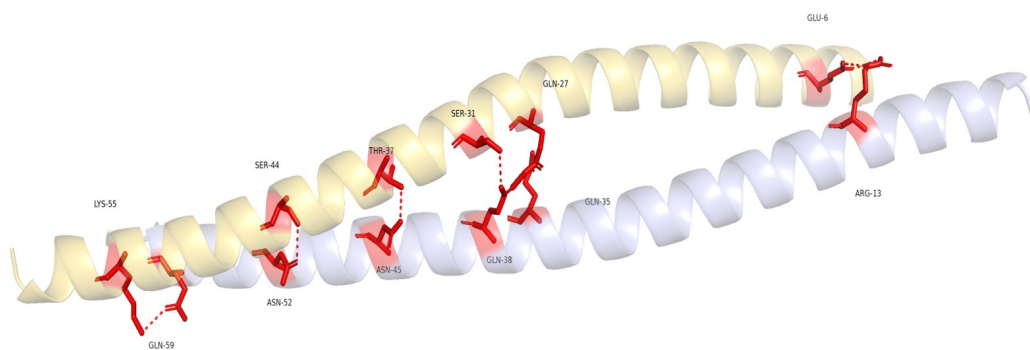


Fig. 9 AlphaFold3 predicts SNARE coiled-coil peptide interactions. (A)(B)(C)(D)

statistically indistinguishable from those of the empty vector control and the parental astaxanthin-producing strain. These results demonstrate that heterologous expression of the SNARE-derived coiled-coil peptides, whether as independent modules or as functional fusions with CrtZ and CrtW, does not exert detectable cytotoxic or growth-inhibitory effects on *E. coli* under the conditions tested(Fig.6).

This observation underscores the biocompatibility of these engineered interaction modules and supports their suitability as molecular tools for metabolic pathway engineering without compromising host fitness.

4 Discussion

In this study, we repurposed α -helical coiled-coil domains from yeast SNARE proteins as modular linkers for the spatial organization of a heterologous biosynthetic pathway. Three lines of evidence support the utility of this strategy. First, multiple sequence alignment of yeast SNARE coiled-coil regions revealed strongly conserved heptad-repeat residues, and AlphaFold3 predicted specific interactions between selected t-SNARE/v-SNARE peptide pairs with consistently high ipTM scores. Second, *in vivo* co-expression in *E. coli* produced co-localized punctate foci of the cognate eGFP- and mCherry-tagged peptides, providing direct visual evidence that these isolated coiled-coil segments retain their native binding specificity even when removed from the full-length proteins and expressed in a heterologous bacterial host. Third, tethering the astaxanthin pathway enzymes CrtZ and CrtW through SNARE peptide pairs increased the astaxanthin titer by 2-fold over the unassembled parent strain, consistent with a substrate-channeling-type mechanism arising from the enforced spatial proximity of the two enzymes.

SNARE-derived coiled-coil peptides expand and complement the existing toolbox of orthogonal interaction modules for synthetic enzyme assembly. Compared with peptide tag pairs such as SpyTag–SpyCatcher (Hatlem et al., 2019; Reddington and Howarth, 2015) or short helical pairs such as

273 RIDD–RIAD, the SNARE coiled-coils offer several distinguishing features. They form a noncovalent,
274 structurally well-characterized four-helix bundle whose stoichiometry, register, and orientation can
275 in principle be tuned by altering helix length or by introducing electrostatic substitutions at the
276 e/g positions of the heptad repeats. Their predicted binding free energy is high enough to ensure
277 stable assembly under physiological conditions, yet they remain noncovalent and therefore reversible,
278 which contrasts with the irreversible isopeptide-bond chemistry of SpyTag–SpyCatcher. Furthermore,
279 because yeast encodes a large set of t- and v-SNAREs with distinct cognate partners, this family
280 is well suited to the future development of orthogonal multi-component assemblies, in a manner
281 analogous to PKS docking domains repurposed for cascade enzyme assembly (Sun et al., 2022).

282 Mechanistically, the 2-fold improvement in astaxanthin titer most likely reflects a combination of
283 effects rather than substrate channeling in its strict, tunnel-mediated sense. CrtZ and CrtW catalyse
284 sequential hydroxylation and ketolation steps on a hydrophobic carotenoid intermediate that parti-
285 tions into the bacterial membrane; co-localization of the two enzymes by SNARE coiled-coil pairing
286 is therefore expected to raise the local effective concentration of the shared intermediate, suppress its
287 loss to competing reactions, and shorten the diffusional path between active sites. Notable variability
288 was observed among the three t-SNARE peptides tested, with Z-vam3-W-Nyv1 outperforming the
289 Vam7- and Vti1-based assemblies. This is consistent with subtle differences in the predicted geom-
290 etry, orientation, or binding affinity of each coiled-coil pair, all of which can influence the relative
291 positioning of the CrtZ and CrtW active sites within the assembled complex. Structural character-
292 ization of the assembled CrtZ–CrtW complexes, together with systematic variation of linker length
293 and rigidity, will be needed to delineate the precise determinants of pathway performance.

294 An important practical consideration for any heterologous interaction module is its impact on the
295 physiology of the production host. Growth curve analysis revealed that none of the SNARE coiled-coil
296 fusion constructs perturbed the growth kinetics of *E. coli* relative to the empty vector or unassembled
297 parent strain. This finding indicates that the SNARE coiled-coil domains, despite originating from
298 a phylogenetically distant eukaryote, do not engage in detectable off-target interactions with native
299 *E. coli* proteins under the conditions tested, in line with their structural orthogonality to bacterial
300 protein–protein interaction networks. This biocompatibility is a prerequisite for scalable application in
301 microbial cell factories, where any growth penalty would directly compromise volumetric productivity.

302 Several aspects of this work invite further investigation. First, although confocal imaging clearly
303 demonstrates assembly, the stoichiometry, size, and dynamic behaviour of the resulting CrtZ–CrtW
304 complexes in living cells remain to be quantified, for example by fluorescence correlation spectroscopy
305 or single-particle tracking. Second, the present study focused on a single t-SNARE/v-SNARE pair
306 per assembly; combining orthogonal coiled-coil pairs should enable the construction of higher-order,

multi-enzyme nanoclusters that recruit additional pathway enzymes such as IPP isomerase or geranyl-geranyl diphosphate synthase to further increase metabolic flux. Third, introducing phosphorylation sites or other regulatory cues into the coiled-coil interface could in principle convert these modules into dynamic switches that respond to intracellular cues such as the ATP/ADP ratio or pH, paving the way toward responsive, self-regulating biosynthetic systems. Finally, given the strong conservation of SNARE coiled-coil motifs across eukaryotes, the strategy described here should be readily transferable to industrially relevant hosts such as *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Corynebacterium glutamicum* for the production of diverse high-value natural products.

In summary, we have established yeast SNARE-derived coiled-coil peptides as a previously unexploited, biocompatible, and programmable family of interaction modules for the modular assembly of metabolic enzymes. Applied to the astaxanthin biosynthetic pathway in *E. coli*, this strategy increased the product titer by 2-fold without compromising host fitness, validating SNARE coiled-coils as a versatile addition to the synthetic biology toolbox. We anticipate that broad adoption of this platform will accelerate the rational design of spatially organized multi-enzyme systems for the efficient biomanufacture of pharmaceuticals, nutraceuticals, and other high-value metabolites.

Conclusion

The present study establishes yeast SNARE-derived coiled-coil peptides as a new class of programmable interaction modules for synthetic enzyme assembly. Conserved coiled-coil domains were first identified by sequence analysis and their interaction specificity was predicted using AlphaFold3, followed by experimental validation in *E. coli* through fluorescence co-localization. By exploiting these orthogonal peptide pairs to spatially organize the astaxanthin biosynthetic enzymes CrtZ and CrtW, we achieved a maximum astaxanthin yield of 1 mg g⁻¹ DCW, representing a 2-fold increase compared with the unassembled control, without imposing detectable growth defects on the host. These findings demonstrate that SNARE-derived coiled-coil domains effectively promote enzyme proximity and enhance metabolic flux while maintaining excellent biocompatibility. Compared with existing modular assembly systems, the SNARE-based strategy offers a large repertoire of naturally evolved interaction pairs with high specificity, modularity, and potential orthogonality, providing a versatile platform for constructing higher-order enzyme assemblies. Beyond astaxanthin biosynthesis, this approach should be broadly applicable to diverse microbial cell factories and metabolic pathways requiring efficient substrate transfer. Future efforts integrating multiple orthogonal SNARE pairs with dynamic regulatory elements are expected to enable programmable spatial organization of complex biosynthetic networks, further expanding the synthetic biology toolbox for sustainable biomanufacturing.

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Author Contributions

Jiankun Lin: Formal analysis, Investigation, Writing – original draft, Writing – review and editing.
Lexiang Jin: Formal analysis, Investigation. Boyang Lin: Formal analysis. Hongwen Li: Supervision,
Writing – review and editing. Qin Song: Formal analysis, Writing – review and editing. Linyuan
Zou: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision,
Writing – review and editing.

Competing Interests

The authors declare no competing interests.

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Availability of Data and Materials

All data supporting the findings of this study are available within the article and its supplementary
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