

Construction and Engineering of Large Biochemical Pathways via DNA Assembler

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Abstract

DNA assembler enables rapid construction and engineering of biochemical pathways in a one-step fashion by exploitation of the in vivo homologous recombination mechanism in *Saccharomyces cerevisiae*. It has many applications in pathway engineering, metabolic engineering, combinatorial biology, and synthetic biology. Here we use two examples including the zeaxanthin biosynthetic pathway and the aureothin biosynthetic gene cluster to describe the key steps in the construction of pathways containing multiple genes using the DNA assembler approach. Methods for construct design, pathway assembly, pathway confirmation, and functional analysis are shown. The protocol for fine genetic modifications such as site-directed mutagenesis for engineering the aureothin gene cluster is also illustrated.

Key words DNA assembler, In vivo homologous recombination, Pathway engineering, Synthetic biology, Metabolic engineering, Gene cluster characterization and engineering

1 Introduction

Methods that enable rapid construction and engineering of biochemical pathways are invaluable in pathway engineering, metabolic engineering, combinatorial biology, and synthetic biology [1–6]. In all these studies, the conventional multistep sequential cloning method is typically used, which includes primer design, PCR amplification, restriction digestion, in vitro ligation, and transformation [7, 8]. In many cases, multiple plasmids are often required [7, 8]. This method is not only time-consuming and inefficient but also relies on unique restriction sites that become limited for large recombinant DNA molecules.

Thanks to its high efficiency and ease to work with, in vivo homologous recombination in yeast has been widely used for gene cloning, plasmid construction, and library creation [9–12]. Recently, we developed a new method, called “DNA assembler,” which enables design and rapid construction of large biochemical pathways in a one-step fashion by exploitation of the in vivo

homologous recombination mechanism in *S. cerevisiae* [13]. This method is highly efficient and circumvents many potential problems associated with conventional cloning method such as requirement of time-consuming, multistep procedures, low efficiency, and dependence on unique restriction sites. Therefore, it represents a versatile approach for construction of biochemical pathways for synthetic biology, metabolic engineering, and functional genomics studies.

In addition to assembling biochemical pathways, DNA assembler also offers unprecedented flexibility and versatility in characterizing and engineering natural product gene clusters [14]. Microorganisms and plants have evolved to produce a myriad array of complex molecules known as natural products or secondary metabolites that are of biomedical and biotechnological importance [15–17]. Sequenced genomes and metagenomes represent a tremendously rich source for discovery of novel pathways involved in natural product biosynthesis [18, 19]. Over the last 2 decades, the complete genome sequences of more than 1,900 organisms have been determined, with more than 10,000 organisms in the pipeline (<http://www.genomesonline.org/cgi-bin/GOLD/bin/gold.cgi>). However, only a tiny fraction of the biosynthetic pathways from these organisms have been characterized, and discovery and sustainable production of natural products are often hampered by our limited ability to manipulate the corresponding biosynthetic pathways. Using DNA assembler, we developed a genomics-driven, synthetic biology-based strategy for rapid characterization and engineering of natural product biosynthetic pathways [14].

Here we use the zeaxanthin biosynthetic pathway and the aureothin biosynthetic cluster as two examples to illustrate the experimental procedures. Figure 1 shows the scheme for constructing the zeaxanthin biosynthetic pathway. Briefly, for each individual gene in the zeaxanthin pathway, an expression cassette including a promoter, a structural gene, and a terminator is PCR-amplified and assembled using overlap extension PCR (OE-PCR) [20]. The 5' end of the first gene expression cassette is designed to overlap with a vector, while the 3' end is designed to overlap with the second cassette. Each successive cassette is designed to overlap with the two flanking ones and the 3' end of the last cassette overlaps with the vector. All overlaps are designed to be at least 50 bp for efficient *in vivo* homologous recombination (*see Note 1*). The resulting multiple expression cassettes are co-transformed into *S. cerevisiae* with the linearized vector through electroporation, which allows the entire pathway to be assembled into a vector. Restriction digestion or DNA sequencing is subsequently used to verify the correctly assembled pathway, after which the cells carrying the correct construct are checked for zeaxanthin production.

For engineering the aureothin gene cluster, as shown in Fig. 2, pathway fragments encoding the aureothin biosynthetic pathway

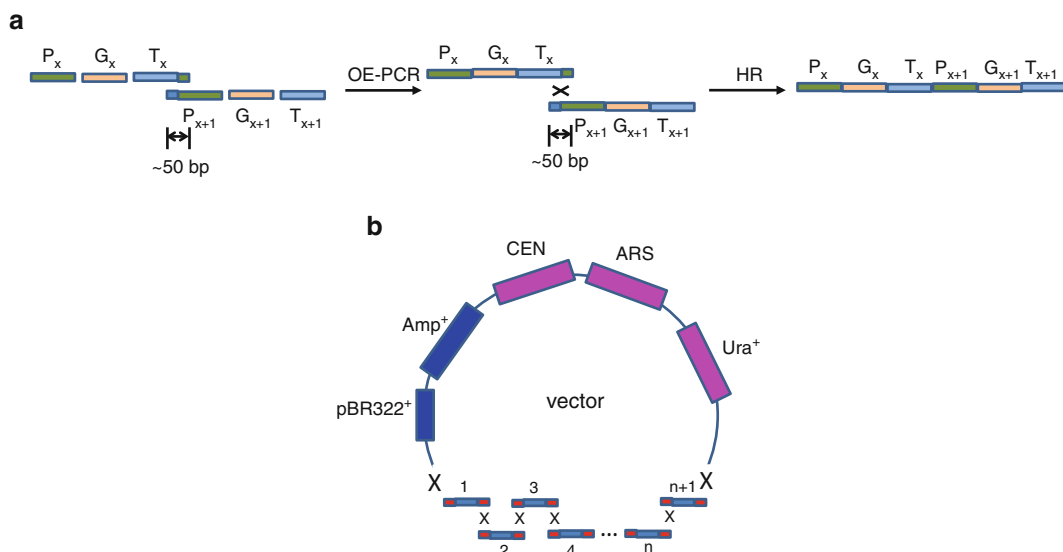


Fig. 1 (a) Preparation of each gene expression cassette using OE-PCR. Promoters (P_x , P_{x+1}), genes (G_x , G_{x+1}), and terminators (T_x , T_{x+1}) are individually PCR-amplified and joined together by OE-PCR. The resulting two cassettes are fused through the in vivo homologous recombination (HR) process. To generate an overlap of approximately 50 bp, the reverse primer used to amplify T_x contains a sequence of the first 20–25 nucleotides of P_{x+1} , and the forward primer used to amplify P_{x+1} contains a sequence of the last 20–25 nucleotides of T_x . **(b)** One-step method for assembly of a biochemical pathway using in vivo homologous recombination (HR) in *S. cerevisiae*

are amplified from the isolated genomic DNA of the native aureothin producer *Streptomyces thioluteus*. The helper fragments carrying the genetic elements needed for DNA maintenance and replication in *S. cerevisiae*, *E. coli*, and the target heterologous expression host *Streptomyces lividans* (see **Note 2**) are amplified from the corresponding vectors. Since PCR primers are designed to generate an overlap region between two adjacent fragments (see **Note 3**), these fragments will be assembled into a single DNA molecule in *S. cerevisiae* through homologous recombination after co-transformation. The isolated plasmids are transformed into *E. coli* for plasmid enrichment and verification, and the correct construct is transformed into *S. lividans* for heterologous expression of the aureothin biosynthetic pathway. Of special note, because the pathway fragments can be readily modified by PCR, various sophisticated genetic manipulations such as point mutagenesis and scarless gene substitution and deletion can be easily performed to confirm gene function, locate key amino acid residues, study biosynthetic mechanisms, express biosynthetic pathways heterologously, and generate new derivatives (see **Note 4**).

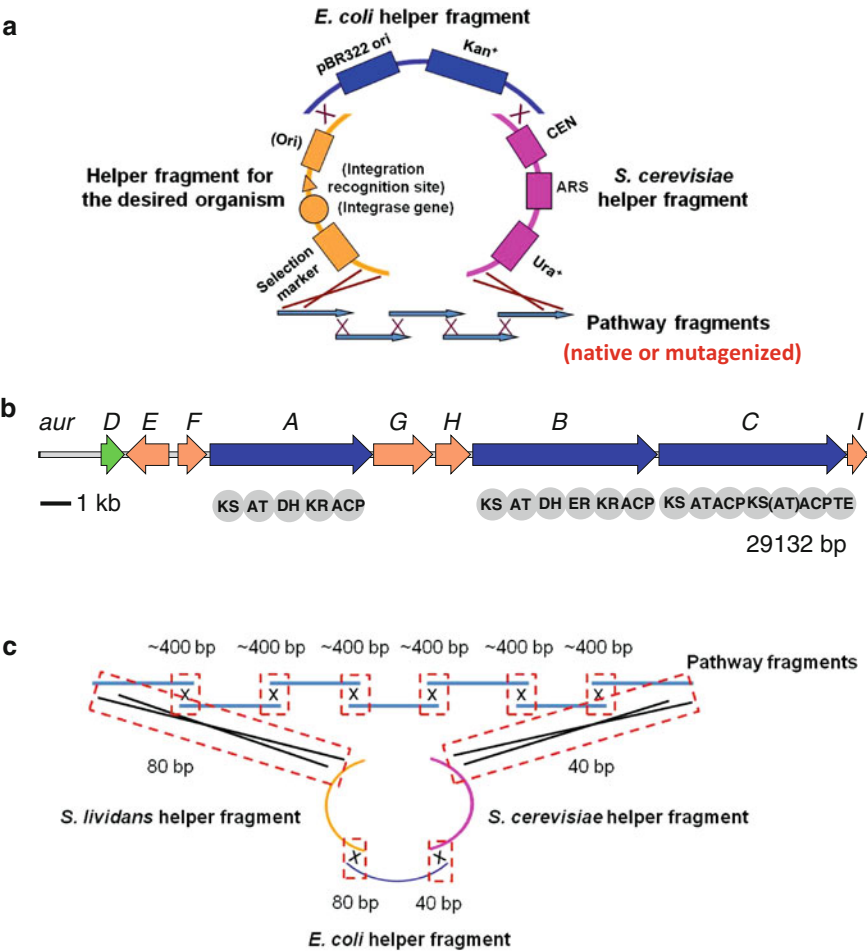


Fig. 2 (a) The DNA assembler-based strategy for efficient manipulation of natural product biosynthetic pathways. Various genetic modifications are introduced in the pathway fragments to be assembled. (b) The aureothin biosynthetic gene cluster from *S. thioluteus*. (c) The overlap lengths between adjacent fragments in assembly

2 Materials

Prepare all solutions using ultrapure water, prepared by purifying deionized water to attain a resistivity of 18.2 mΩ cm at 25 °C. Prepare and store all reagents at room temperature unless indicated otherwise.

2.1 DNA Preparation

1. pRS416: Obtained from New England Biolabs (Beverly, MA, USA) and serves as the template for amplifying the *S. cerevisiae* helper fragment for assembling the aureothin gene cluster variants.

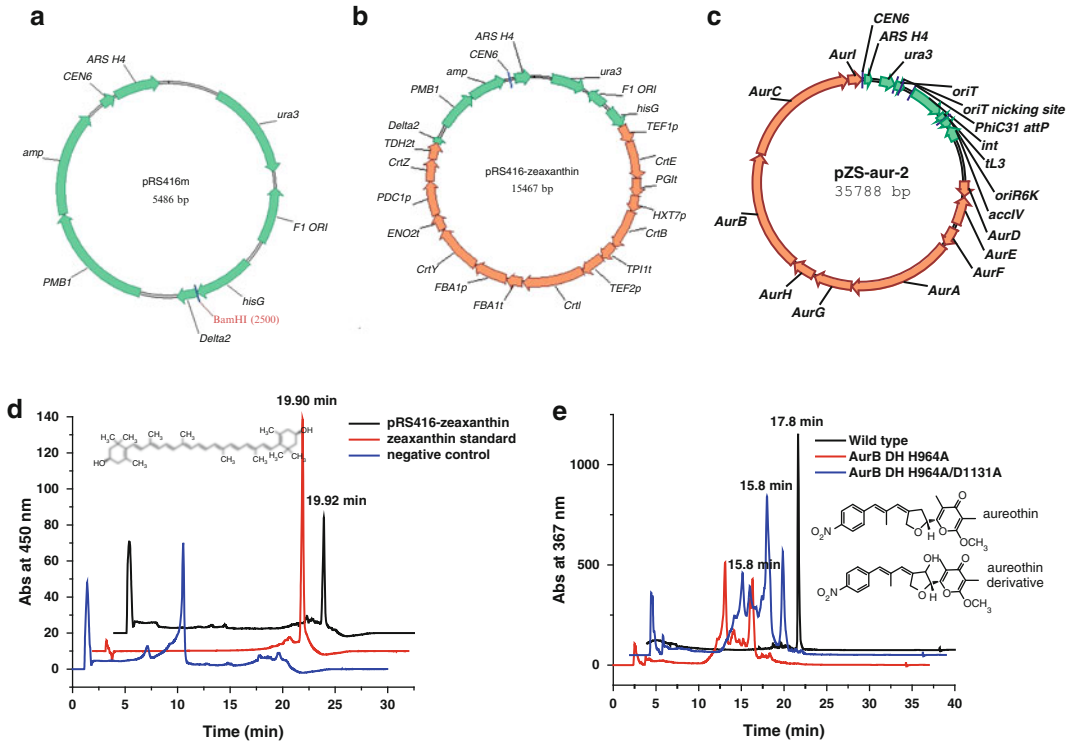


Fig. 3 (a) The vector map of pRS416m. (b) The vector map of the construct pRS416m-zeaxanthin. *CEN6*: centromere; *ARS H4*: automatic replication sequence; *ura3*: selection marker in *S. cerevisiae*; *amp*: selection marker in *E. coli*; *hisG* and *delta2*: two regions flanking the zeaxanthin biosynthetic pathway; *PMB1*: *E. coli* origin of replication. *F1 ORI*: this region is contained in the original pRS416 vector, but is not required for the construction of the plasmid containing the zeaxanthin biosynthetic pathway. (c) The vector map of the construct pZS-aur-2. *oriR6K*: *E. coli* origin of replication; *accIV*: apramycin resistance gene (it can be used as the selection marker in both *Streptomyces* and *E. coli*); *oriT*: conjugal transfer ori; *PhiC31 attP*: the ϕ C31 recognition site; *int*: the ϕ C31 integrase; *tL3*: a terminator. (d) HPLC analysis of the cell extracts from *S. cerevisiae* carrying the zeaxanthin biosynthesis pathway. Cells carrying the empty vector pRS416 were used as the negative control. Zeaxanthin structure was shown. (e) LC-MS analysis of the *S. lividans* clones carrying the wild type aureothin biosynthetic pathway and the mutant aureothin biosynthetic pathways. The structures of aureothin and its derivative were shown

2. pRS416m: pRS416 with a *hisG* sequence and a *delta2* [21] sequence that flank the multiple cloning site and serves as the vector for assembly of the zeaxanthin pathway (Fig. 3a) (see Note 5).
3. pCAR- Δ CrtX: It contains the genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from *Erwinia uredovora* for zeaxanthin biosynthesis (Prof. E.T. Wurtzel, City University of New York, NY, USA) [22–24].
4. *S. thioluteus* strain: Obtained from the American Tissue Culture Collection (ATCC 12310, Manassas, VA, USA).

5. The genomic DNA of *S. thioluteus*: Isolated from *S. thioluteus* using Wizard Genomic DNA Isolation Kit (Promega, Madison, WI, USA).
6. pAE4: A *Streptomyces*–*E. coli* shuttle vector obtained from Professor William Metcalf (University of Illinois, Urbana, IL, USA) and serves as the template for amplifying the *S. lividans* helper fragment and the *E. coli* helper fragment for assembly of the aureothin gene cluster (the complete sequence of the plasmid can be obtained by request from the authors).
7. *E. coli* strain WM6026: Obtained from Professor William Metcalf and serves as the donor strain for conjugating plasmids to *S. lividans* (the details of strain construction can be obtained by request from the authors).
8. 0.5 M ethylenediamine tetraacetic acid (EDTA) solution (pH 8.0): For a 500 mL of stock solution of 0.5 M EDTA, weigh out 93.05 g of EDTA disodium salt (MW = 372.2) and dissolve it in 400 mL of deionized water. Adjust to pH 8.0 with NaOH and correct the final volume to 500 mL. EDTA will not be dissolved completely in water unless the pH is adjusted to about 8.0.
9. Concentrated stock solution of TAE (50×): Weigh 242 g of Tris base (MW = 121.14) and dissolve it in approximately 750 mL of deionized water. Carefully add 57.1 mL of glacial acid and 100 mL of 0.5 M EDTA, and adjust the solution to a final volume of 1 L. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.
10. Working solution of TAE buffer (1×): Dilute the stock solution by 50-fold with deionized water. Final solute concentrations are 40 mM Tris acetate and 1 mM EDTA.
11. 0.7 % agarose gel in 1× TAE buffer: Add 0.7 g of agarose into 100 mL of 1× TAE buffer and microwave until agarose is completely melted. Cool the solution to approximately 70–80 °C. Add 5 µL of ethidium bromide into the solution and mix well. Pour 25–30 mL of solution onto an agarose gel rack with appropriate 2-well or 8-well combs.
12. Wizard Genomic DNA Isolation Kit (Promega, Madison, WI, USA).
13. QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA).
14. QIAprep Miniprep Kit (QIAGEN, Valencia, CA, USA).
15. DNA polymerase: Any polymerase with high fidelity can be used.
16. Failsafe PCR 2× Premix G: Containing dNTPs and PCR buffer (EPICENTRE Biotechnologies, Madison, WI, USA).

17. 5× Phusion GC Reaction Buffer (New England Biolabs, Beverly, MA, USA).
18. 40× dNTPs premix: 10 mM each nucleotide.
19. Dimethyl sulfoxide (DMSO).
20. *Bam*HI restriction enzyme (New England Biolabs, Ipswich, MA, USA).
21. 3 M sodium acetate pH 5.0: Weigh 12.3 g of sodium acetate (MW = 82.03) and dissolve it into 50 mL of deionized water. Adjust to pH 5.0 by HCl.
22. 10 mg/mL glycogen: Dissolve 10 mg of glycogen in 1 mL of deionized water.
23. NanoDrop2000c: Used to measure the concentration of DNA and check the OD₆₀₀ of the cells (Thermo Scientific, Wilmington, DE, USA).
24. Benchtop centrifuges to separate cells and supernatant.
25. Molecular imager gel doc: Used to check DNA on the agarose gel (Bio-Rad, Hercules, CA, USA).

2.2 Transformation

1. *S. cerevisiae* HZ848 (*MATα*, *ade2-1*, *Δura3*, *his3-11, 15, trp1-1*, *leu2-3, 112*, *can1-100*): Used as the host for DNA assembly (see Note 6).
2. YPAD medium: Dissolve 6 g of yeast extract, 12 g of peptone, 12 g of dextrose, and 60 mg of adenine hemisulfate in 600 mL of deionized water. Autoclave at 121 °C for 15 min.
3. Synthetic complete drop-out medium lacking uracil (SC-Ura): Dissolve 3 g of ammonium sulfate, 1 g of yeast nitrogen source without ammonium sulfate and amino acids, 0.5 g of complete synthetic medium minus uracil (CSM-Ura; MP Biomedicals, Solon, OH, USA), 26 mg of adenine hemisulfate, and 12 g of dextrose in 600 mL of deionized water, and adjust the pH to 5.6 by NaOH. Autoclave at 121 °C for 15 min.
4. SC-Ura-agar: SC-Ura and 20 g/L of agar.
5. 1 M sorbitol solution: Dissolve 91.1 g of sorbitol (MW = 182.17) in 400 mL of deionized water and adjust to a final volume of 500 mL. Sterilize the solution by filtering it through a filter with a pore size of 0.22 μm.
6. Gene Pulser II and Pulse controller plus: Used to transform plasmids into *S. cerevisiae* and *E. coli* through electroporation (Bio-Rad, Hercules, CA, USA).

2.3 Verification of the Clones

1. Zymoprep II yeast plasmid miniprep (Zymo Research, Orange, CA, USA).
2. *E. coli* strain BW25141 (*F*⁻, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, *Δ(phoB-phoR)580*, *λ*⁻, *galU95*, *ΔuidA3::pir*⁺, *recA1*,

endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR514: Work as a standard cloning strain (obtained from Professor William Metcalf at the University of Illinois, Urbana, IL).

3. *ScaI*, *PstI*, *SacI*, and *AscI* restriction enzymes (New England Biolabs, Ipswich, MA, USA) (*see* **Note 7**).
4. 1 M glucose solution: Dissolve 90 g of D-glucose in 400 mL of deionized water and adjust to a final volume of 500 mL. Filter-sterilize it.
5. SOC medium: Add 20 g of bacto-tryptone, 5 g of yeast extract, 0.5 g of NaCl, and 186.4 mg of KCl into 980 mL of deionized water. Adjust the pH to 7.0 with NaOH. Autoclave at 121 °C for 15 min. After the solution cools down to 70–80 °C, add 20 mL of sterile 1 M glucose.
6. 100 mg/mL ampicillin stock solution: Dissolve 1 g of ampicillin powder in 10 mL of deionized water and filter-sterilize it.
7. 50 mg/mL apramycin stock solution: Dissolve 0.5 g of apramycin powder in 10 mL of deionized water and filter-sterilize it.
8. LB broth: Add 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl into 1 L of deionized water. Autoclave at 121 °C for 15 min.
9. LB-agar: LB broth and 20 g/L agar.
10. LB-Amp⁺ agar plates: Autoclave LB-agar and when the solution cools down to 70–80 °C, add 1 mL of 100 mg/mL ampicillin to 1 L of LB-agar. Pour 20–25 mL into each Petri dish.
11. LB-Apr⁺ agar plates: Autoclave LB-agar and when the solution cools down to 70–80 °C, add 1 mL of 50 mg/mL apramycin to 1 L of LB-agar. Pour 20–25 mL into each Petri dish.

2.4 Detection of Zeaxanthin

1. 0.1 % trifluoroacetic acid (TFA) buffer: Add 1 mL of TFA into 999 mL of deionized water.
2. 10 µg/mL zeaxanthin standard solution: Dissolve 1 mg of zeaxanthin (Sigma, St Louis, MO, USA) in 100 mL of methanol.
3. French pressure cell press: Used to lyse the yeast cells.
4. Rotary evaporator R-205: Used to evaporate the solvent.
5. High performance liquid chromatography (HPLC) equipment.
6. ZORBAX SB-C18 column (Agilent Technologies, Palo Alto, CA, USA).

2.5 Conjugation of Plasmids into *S. lividans*

1. 3.8 mg/mL 2,6-diaminopimelic acid (DAP) stock solution: Dissolve 38 mg of DAP powder in 10 mL of deionized water and filter-sterilize it.

2. LB- Apr^+ -DAP plates: LB- Apr^+ plates and 19 $\mu\text{g}/\text{mL}$ DAP.
3. A mixture of nalidixic acid and apramycin each at the concentration of 1 mg/mL : Dissolve 20 mg of nalidixic acid powder in 15 mL of deionized water. Nalidixic acid will not be dissolved completely in water unless the pH is adjusted to approximately 12.0. Slowly adjust pH to 9.0–9.5 with 1 M HCl and add 400 μL of 50 mg/mL apramycin. The final pH after supplementing apramycin should drop to approximately 7.8–8.2. Correct the final volume to 20 mL and filter-sterilize it.
4. *S. lividans* spores: Prepared according to the protocol in the book “Practical Streptomyces Genetics,” second edition, pp. 44–47 [25].
5. R2 no-sucrose agar plates: Add 0.25 g of K_2SO_4 , 10.12 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 g of glucose, 0.1 g of Difco casamino acids, and 5.73 g of TES into 990 mL of deionized water. Autoclave at 121 °C for 15 min. After the solution cools down to 70–80 °C, add 1 mL of 5 mg/mL KH_2PO_4 , 8 mL of 36.8 mg/mL $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.5 mL of 0.2 g/mL L-proline, and 0.5 mL of 1 M NaOH (all these solutions need to be filter-sterilized in advance). Pour 20–25 mL into each Petri dish. Please refer to the recipe in the book “Practical Streptomyces Genetics,” p. 408 [25].
6. ISP2- Apr^+ agar plates: Add 10 g of malt extract, 4 g of yeast extract, 4 g of glucose, and 20 g of agar into 1 L of deionized water. Adjust pH to 7.2 and autoclave at 121 °C for 15 min. When the solution cools down to 70–80 °C, add 1 mL of 50 mg/mL apramycin. Mix well and pour 20–25 mL into each Petri dish.

2.6 Culturing of the Aureothin Pathway Variants and Detection of Aureothin and Its Derivatives

1. MYG medium: Add 10 g of malt extract, 4 g of yeast extract, and 4 g of glucose into 1 L of deionized water. Adjust pH to 7.2 and autoclave at 121 °C for 15 min.
2. Ethyl acetate.
3. 100 $\mu\text{g}/\text{mL}$ aureothin standard solution: Dissolve 1 mg of aureothin (BioAustralis, Smithfield, Australia) in 10 mL of methanol.
4. Agilent 1100 series LC/MSD XCT plus ion trap mass spectrometer (Agilent, Palo Alto, CA).

3 Methods

3.1 DNA Preparation for Assembling the Zeaxanthin Biosynthetic Pathway

1. Amplify the genes *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from the plasmid pCAR- ΔCrtX and amplify the corresponding promoter and terminator from the genomic DNA of *S. cerevisiae* using the primers listed in Table 1. Set up the reaction mixtures as follows: 50 μL of FailSafe PCR 2xPreMix G, 2.5 μL of

Table 1
The primers used in assembling the zeaxanthin pathway in pRS416m

Name	Sequence
hisG-f	GGCCAGTGAGCGCGCGTAATACGACTCACTA TAGGCGCGCCTGCGTGAAGTCGAAG
hisG-r	GGAGTAGAAACATTTTGAAGCTATTTCCAGTCAAT CAGGGTATTG
TEF1p-f	CTTCAATACCCTGATTGACTGGAAATAGCTTCAAAA TGTTTCTACTC
TEF1p-r	GAACGTGTTTTTTTGC GCAGACCGTCATTTTGTA ATTAAAACTTAGATTAG
CrtE-f	CTAATCTAAGTTTTTAATTACAAAATGACGGTCTGCG CAAAAAACACGTTT
CrtE-r	GGTATATATTTAAGAGCGATTTGTTTAACTGACGGCAGCG
PGIt-f	CGCTGCCGTCAGTTAAACAAATCGCTCTTAAATATATACC
PGIt-r	CCGAAATTGTTCTACGAGAAGTGGTATACTGGAGGCTT CATGAGTTATG
HXT7p-f	CATAACTCATGAAGCCTCCAGTATACCACTTCTCGTAGGA ACAATTTTCGG
HXT7p-r	GAGTAACGACGGATTATTCAATTTTTTGATTAAAATTAAAA AAAC
CrtB-f	GTTTTTTTAATTTTAATCAAAAAATGAATAATCCGTCGT TACTC
CrtB-r	GATAATATTTTTATATAATTATATTAATCCTAGAGCGGGC GCTGCCAGAG
TPI1t-f	CTCTGGCAGCGCCCGCTCTAGGATTAATATAATTATATAA AAATATTATC
TPI1t-r	CTATATGTAAGTATACGGCCCTATATAACAGTTGAAATTTGG
TEF2p-f	CCAAATTTCAACTGTTATATAGGGCCGTATACTTACATATAG
TEF2p-r	CACCAATTACCGTAGTTGGTTTCATGTTTAGTTAATTATAGT TCGTTG
CrtI-f	CAACGAACTATAATTAATAAATGAAACCAACTACGGTAA TTGGTG
CrtI-r	CTCATTAAAAACTATATCAATTAATTTGAATTAACTCATATC AGATCCTCCAGCATC
FBA1t-f	GATGCTGGAGGATCTGATATGAGTTAATTCAAATTAATTGATA TAGTTTTTTAATGAG
FBA1t-r	GTTCAAGCCAGCGGTGCCAGTTGGAGTAAGCTACTATGA AAGACTTTAC

(continued)

Table 1
(continued)

Name	Sequence
FBA1p-f	GTAAAGTCTTTCATAGTAGCTTACTCCAACCTGGCACCGC TGGCTTGAAC
FBA1p-r	CAGATCATAATGCGGTTGCATTTTGAATATGTATTACTT GGTTATGG
CrtY-f	CCATAACCAAGTAATACATATTCAAAATGCAACCGCA TTATGATCTG
CrtY-r	CTAATAATTCTTAGTTAAAAAGCACTTTAACGATGAGT CGTCATAATGG
ENO2t-f	CCATTATGACGACTCATCGTTAAAGTGCTTTTAACT AAGAATTATTAG
ENO2t-r	GGAACATATGCTCACCCAGTCGCATGAGGTATCAT CTCCATCTCCCATATG
PDC1p-f	CATATGGGAGATGGAGATGATACCTCATGCGACTGGG TGAGCATATGTTCC
PDC1p-r	GGCATTCCAAATCCACAACATTTTGATTGATTGACTGTG
CrtZ-f	CACAGTCAAATCAATCAAAATGTTGTGGATTGGAATGCC
CrtZ-r	CATTAAAGTAACTTAAGGAGTTAAATTTACTTCCCGGATGC GGGCTC
TDH2t-f	GAGCCCGCATCCGGGAAGTAAATTTAACTCCTTAAGTT ACTTTAATG
TDH2t-r	GATCCGTTAGACGTTTCAGCTTCCAGCGAAAAGCCAA TTAGTGTGATAC
Delta2-f	GTATCACACTAATTGGCTTTTCGCTGGAAGCTGAA ACGTCTAACGGATC
Delta2-r	TTACGCCAAGCGCGCAATTAACCCTCACTAAAGGCG CGCCGAGAACTTCTAGTATATTC

forward primer (20 pmol/ μ L), 2.5 μ L of reverse primer (20 pmol/ μ L), 1 μ L of template (10–50 ng of *S. cerevisiae* genomic DNA or the plasmid pCAR- Δ CrtX), 1 μ L of DNA polymerase, and 43 μ L of ddH₂O in a total volume of 100 μ L.

2. PCR condition: Fully denature at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.
3. Load the 100 μ L of PCR products onto 0.7 % agarose gels and perform electrophoresis at 120 V for 20 min.
4. Gel-purify PCR products using the QIAquick Gel Extraction Kit.

5. Check the concentrations of the purified products using NanoDrop.
6. Perform OE-PCR to generate each gene expression cassette (*see Note 8*). Set up the first-step reaction mixture as follows: 10 μL of FailSafe PCR 2xPreMix G, 100 ng of promoter fragment, 100 ng of *crt* gene fragment, 100 ng of terminator fragment, and 0.2 μL of DNA polymerase. Add ddH₂O to a final volume of 20 μL .
7. Reaction condition: Fully denature at 98 °C for 30 s, followed by 10 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.
8. Set up the second-step reaction mixture as follows: 50 μL of FailSafe PCR 2 $\times$ PreMix G, 10 μL of first-step reaction mixture, 1 μL of DNA polymerase, 2.5 μL of forward primer (20 pmol/ μL), 2.5 μL of reverse primer (20 pmol/ μL), and 34 μL ddH₂O in a total volume of 100 μL .
9. Reaction condition: Fully denature at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.
10. Digest pRS416m by *Bam*HI at 37 °C for 3 h. Digestion condition: 5 μL of 10 $\times$ buffer, 0.5 μL of 100 $\times$ BSA, 3 μg of pRS416m, and 30 units of *Bam*HI. Add ddH₂O to a final volume of 50 μL .
11. Load the PCR and digestion products onto 0.7 % agarose gels and perform electrophoresis at 120 V for 20–30 min.
12. Gel-purify the PCR and digestion products using the QIAquick Gel Extraction Kit.
13. Check the concentrations of the purified products using NanoDrop.
14. Take 200–300 ng of each fragment, mix in a tube, and calculate the final volume.
15. Add 10 % v/v 3 M sodium acetate and 2 % v/v 10 mg/mL glycogen (e.g., if there is 100 μL of mixture, add 10 μL of sodium acetate and 2 μL of glycogen) and mix well.
16. Add 2 $\times$ v/v 100 % ethanol (e.g., if the final volume is about 110 μL , add 220 μL ethanol) and mix well.
17. Store the DNA mixture at –80 °C for at least an hour.
18. Centrifuge at 4 °C, 16,100 $\times g$ for 20 min. Usually the precipitated DNA can be seen at the bottom of the tube.
19. Remove the supernatant completely (do not touch the DNA).
20. Add 500 μL of 70 % ethanol to wash the DNA pellet, and centrifuge at room temperature, 16,100 $\times g$ for 3 min.

21. Remove the ethanol completely and air dry the pellet for 1–2 min (do not overdry it).
22. Resuspend the DNA pellet in 4 μL of ddH₂O. Now the DNA is ready for transformation (*see Note 9*).

3.2 DNA Preparation for Assembling the Aureothin Gene Cluster Variants

1. Amplify the aureothin biosynthetic pathway fragments from the genomic DNA of *S. thioluteus* and amplify the *S. cerevisiae*, *E. coli*, and *S. lividans* helper fragments from the corresponding vectors using the primers listed in Table 2. For constructing the mutant pathways, mutations are added into the corresponding primers. Set up the reaction for amplifying the *S. cerevisiae* helper fragment as follows: 20 μL of Buffer GC, 2.5 μL of dNTP premix, 2.5 μL of forward primer (20 pmol/ μL), 2.5 μL of reverse primer (20 pmol/ μL), 1 μL of template pRS416 at the concentration of 50–100 ng/ μL , 1 μL of DNA polymerase, and 70.5 μL of ddH₂O in a total volume of 100 μL (*see Note 10*). Set up all the other reaction mixtures as follows: 50 μL of FailSafe PCR 2 $\times$ PreMix G, 2.5 μL of forward primer (20 pmol/ μL), 2.5 μL of reverse primer (20 pmol/ μL), 1 μL of template (10–50 ng of *S. thioluteus* genomic DNA or the plasmid pAE4), 1 μL of DNA polymerase, 5 μL of DMSO (*see Note 11*), and 38 μL of ddH₂O in a total volume of 100 μL .
2. PCR condition for amplifying the *S. cerevisiae* helper fragment: Fully denature at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 45 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min (*see Note 10*). PCR condition for all the other fragments: Fully denature at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 1–3 min, with a final extension at 72 °C for 10 min (*see Note 11*).
3. Load the 100 μL of PCR products onto 0.7 % agarose gels and perform electrophoresis at 120 V for 20 min.
4. Gel-purify PCR products using the QIAquick Gel Extraction Kit.
5. Check the concentrations of the purified products using NanoDrop.
6. Take 200–300 ng of each fragment, mix in a tube, and calculate the final volume.
7. Add 10 % v/v 3 M sodium acetate and 2 % v/v 10 mg/mL glycogen (e.g., if there is 100 μL of mixture, add 10 μL of sodium acetate and 2 μL of glycogen) and mix well.
8. Add 2 $\times$ v/v 100 % ethanol (e.g., if the final volume is about 110 μL , add 220 μL ethanol) and mix well.
9. Store the DNA mixture at –80 °C for at least an hour.
10. Centrifuge at 4 °C, 16,100 $\times g$ for 20 min. Usually the precipitated DNA can be seen at the bottom of the tube.

Table 2
The primers used in assembling the aureothin gene cluster variants

Name	Sequence
Aur-1-for	TGGTACTGCAAATACGGCATCAGTTACCG TGAGCAGATCGGATCAGCTCGTCCCGTTTCGG
Aur-1-rev	GCTGCTCTTCTCGCATCGTC
Aur-2-for	CGTAGAGGAGCTCCAGCAGC
Aur-2-rev	CTCCTCCAGCACCTCGCAGC
Aur-3-for	TCCTGACCTTCGACTCGCTG
Aur-3-rev	CATGTTTCGATCCTTCCGTTG
Aur-4-for	GACGGTGCACCAGCTGGTCA
Aur-4-rev	GTTGCCGGTCATGTGGTAGC
Aur-5-for	ATGACCAATGACGCCAAGAC
Aur-5-rev	CCGTCCATCAGGTCTGAACGC
Aur-6-for	CCTACTACGGCCTGGTGGAC
Aur-6-rev	CATCGCCGTCATCGAGACGA
Aur-7-for	GCAACGAAGGACATGTCCAG
Aur-7-rev	TTATAGCACGTGATGAAAAGGACCCAGGTGGCACTTTTCGTC AGTCAGTCGTCCAGGCGC
Yeast-for	CGAAAAGTGCCACCTGGGTC
Yeast-rev	AATATTGTGAGTTTAGTATACATGCA
Strep-for	GTATTATAAGTAAATGCATGTATACTAAACTCACAAATATTATGGCGC GCCGACGTGCTCA
Strep-rev	ATTAGCCATGGCATCACAGTATCGTGATGACATTAATTAACGCAATC CAGTGCAAAGCTA
<i>E. coli</i> -for	AGACGAAGAAGCTAGCTTTGCACTGGATTGCGTTAATTAAT GTCATCACGATACTGTGAT
<i>E. coli</i> -rev	GCCCATGACCACCGTCGTCTCCGAACGGGACGAGCTGATC CGATCTGCTCACGGTAACTG
AurB DH H964A-for	TGTCGGCGCGGACCGAGTCCTGGCTGGCCGAC <u>GCC</u> GTCGTGCT CGGCTCCACGCTCGTCC
AurB DH H964A-rev	GGACGAGCGTGAGACCGAGCACGAC <u>GCG</u> CTCGGCCAGCCAGGA CTCGGTCCGCGCCGACA
AurB DH D1131A-for	GCTACGGCGTCCACCCCGCGTCTCTC <u>GCC</u> GCCGCACTGCACACC GCCCTCCTGAAGGAGG
AurB DH D1131A-rev	CCTCCTTCAGGAGGGCGGTGTGCAGTGCGGCG <u>GCG</u> GAGGAGCGCG GGGTGGACCCGTAGC

The mutated codons are underlined

11. Remove the supernatant completely (do not touch the DNA).
12. Add 500 μL of 70 % ethanol to wash the DNA pellet, and centrifuge at room temperature, $16,100 \times g$ for 3 min.
13. Remove the ethanol completely and air dry the pellet for 1–2 min (do not overdry it).
14. Resuspend the DNA pellet by 4 μL of ddH₂O. Now the DNA is ready for transformation (*see* **Note 9**).

3.3 Transformation

1. Inoculate a single colony of HZ848 into 3 mL of YPAD medium and grow overnight in a shaker at 30 °C and 250 rpm.
2. Measure the OD₆₀₀ of the seed culture and inoculate the appropriate amount to 50 mL of fresh YPAD medium to obtain an OD₆₀₀ of 0.2 (e.g., if the overnight culture has an OD₆₀₀ of 10, then add 1 mL into 50 mL of fresh YPAD medium).
3. Continue growing the 50 mL of culture for approximately 4 h to obtain an OD₆₀₀ of 0.8 (*see* **Note 12**).
4. Spin down the yeast cells at 4 °C, $3,220 \times g$ for 10 min and remove the spent medium.
5. Use 50 mL of ice-cold ddH₂O to wash the cells once and centrifuge again.
6. Discard water, add 1 mL of ice-cold ddH₂O to resuspend the cells, and move them to a sterile Eppendorf tube.
7. Spin down the cells using a bench top centrifuge for 30 s at 4 °C, $4,500 \times g$.
8. Remove water and use 1 mL of 1 M ice-cold sorbitol to wash the cells once (now the cells look slightly yellow). Centrifuge again and remove the sorbitol.
9. Resuspend the cells in 250–300 μL of chilled 1 M sorbitol and distribute them into 50 μL aliquots.
10. Now each 50 μL of cells is ready for electroporation (*see* **Note 13**). Mix the 4 μL of DNA with 50 μL of yeast cells and put the mixture into a chilled electroporation cuvette.
11. Electroporate the cells at 1.5 kV and quickly add 1 mL of pre-warmed (30 °C) YPAD medium to resuspend cells (*see* **Note 14**).
12. Grow in a shaker at 30 °C, 250 rpm for 1 h.
13. Spin down the cells in a sterile tube at $16,100 \times g$ for 30 s and remove the YPAD medium.
14. Use 1 mL of room temperature sorbitol solution to wash the cells 2–3 times and finally resuspend the cells in 1 mL sorbitol.
15. Spread 100 μL of resuspended cells onto SC-Ura plates.
16. Incubate the plates at 30 °C for 2–3 days until colonies appear.

3.4 Verification of the Correctly Assembled Pathways

1. Randomly pick ten colonies from the SC-Ura plate and inoculate each colony into 1.5 mL of SC-Ura liquid medium. Grow at 30 °C for 1.5 days (*see* **Note 15**).
2. Purify yeast plasmid DNA from each 1.5 mL of culture using the Zymoprep II kit.
3. Mix 2 µL of isolated plasmid DNA with 50 µL of *E. coli* BW25141 cells and put the mixture into a chilled electroporation cuvette (*see* **Note 16**).
4. Electroporate the cells at 2.5 kV and quickly add 1 mL of SOC medium to resuspend the cells (*see* **Note 14**).
5. Grow in a shaker at 37 °C, 250 rpm for 1 h.
6. Spin down the cells, remove 800 µL of SOC medium, resuspend the pellet with the remaining 200 µL of SOC medium, and spread the cells on an LB-Amp⁺ plate for the zeaxanthin construct or on an LB-Apr⁺ plate for the aureothin constructs.
7. Incubate the plates at 37 °C for 16–18 h until colonies appear (*see* **Note 17**).
8. Inoculate a single colony from each plate to 5 mL of LB supplemented with 100 µg/mL ampicillin (the zeaxanthin construct) or 50 µg/mL apramycin (the aureothin constructs), and grow at 37 °C for 12–16 h.
9. Purify *E. coli* plasmids from each 5 mL of culture using the QIAgen Miniprep kit.
10. Check the plasmid concentrations by NanoDrop.
11. Verify the correctly assembled pathway through two separate restriction digestion reactions for each construct (*see* **Note 7**).
12. Digestion condition by *ScaI* at 37 °C for 3 h: 1.5 µL of 10× buffer, 0.15 µL of 100× BSA, 300 ng of plasmid, and 5 units of *ScaI*. Add ddH₂O to a final volume of 15 µL. Expected bands for the assembled zeaxanthin pathway (Fig. 3b): 1,750, 2,131, 2,628, 3,223, 5,735 bp.
13. Digestion condition by *PsiI* at 37 °C for 3 h: 1.5 µL of 10× buffer, 0.15 µL of 100× BSA, 300 ng of plasmid, and 5 units of *PsiI*. Add ddH₂O to a final volume of 15 µL. Expected bands for the assembled zeaxanthin pathway (Fig. 3b): 215, 1,389, 1,689, 1,782, 2,425, 2,752, 5,215 bp.
14. Digestion condition by *SacI* at 37 °C for 3 h: 1.5 µL of 10× buffer, 0.15 µL of 100× BSA, 300 ng of plasmid, and 5 units of *SacI*. Add ddH₂O to a final volume of 15 µL. Expected bands for the assembled aureothin gene cluster variants (Fig. 3c): 163, 696, 752, 839, 1,207, 1,965, 2,370, 2,956, 3,074, 3,612, 3,693, 5,250, 9,211 bp.
15. Digestion condition by *AscI* at 37 °C for 3 h: 1.5 µL of 10× buffer, 0.15 µL of 100× BSA, 300 ng of plasmid, and 5 units of

AscI. Add ddH₂O to a final volume of 15 μ L. Expected bands for the assembled aureothin gene cluster variants (Fig. 3c): 487, 1,890, 2,927, 3,154, 3,716, 5,419, 5,433, 5,810, and 6,952 bp.

3.5 Detection of Zeaxanthin

1. Inoculate a single colony carrying the zeaxanthin biosynthetic pathway into 3 mL of SC-Ura liquid medium and grow at 30 °C, 250 rpm for 1.5 days.
2. Inoculate 2.5 mL of seed culture into 250 mL of fresh SC-Ura medium and continue to grow at 30 °C, 250 rpm for 4 days.
3. Cells are collected by centrifugation at $3,220 \times g$, resuspended with 5 mL of acetone, and lysed by French pressure cell at 10,000 psi.
4. Supernatants are collected after centrifugation at $16,100 \times g$ for 3 min and evaporated to dryness using rotary evaporator.
5. After resuspension in 0.5–1 mL of methanol, 100 μ L of sample is loaded onto the Agilent ZORBAX SB-C18 column and analyzed at 450 nm by HPLC with a 0.5 mL/min flow rate as follows: buffer A: H₂O with 0.1 % TFA, buffer B: 100 % CH₃OH; 0–3 min, 60 % CH₃OH; 3–15 min, linear gradient from 60 % CH₃OH to 100 % CH₃OH; 15–17 min, 100 % CH₃OH; 17–20 min, linear gradient from 100 % CH₃OH to 60 % CH₃OH. Authentic zeaxanthin is used as standard, which was eluted at 19.9 min.

3.6 Conjugation and Heterologous Expression of the Aureothin Pathway Variants in *S. lividans*

1. Mix 50–100 ng of each verified plasmid with 50 μ L of *E. coli* WM6026 cells and put the mixture into a chilled electroporation cuvette.
2. Electroporate the cells at 2.5 kV, and quickly add 1 mL of SOC medium and 5 μ L of 38 mg/mL DAP to resuspend cells (*see* Notes 14 and 18).
3. Grow in a shaker at 37 °C, 250 rpm for 1 h.
4. Spread 100 μ L of each culture on an LB-Apr⁺-DAP plate.
5. Incubate the plates at 37 °C for 16 h until colonies appear.
6. Inoculate a single colony from each plate to 2 mL of LB supplemented with 50 μ g/mL apramycin and 10 μ L of 38 mg/mL DAP, and grow at 37 °C for approximately 2 h until OD₆₀₀ reaches 0.6–0.8.
7. Spin down 100 μ L of cell cultures in an Eppendorf tubes and wash the cell pellets each with 1 mL of fresh LB medium.
8. Spin down the cells and wash one more time.
9. Resuspend the cell pellets each with 1 mL of LB.

10. Mix 2 μL of the resuspended cells with 25 μL of *S. lividans* spores by pipetting and spot 2 μL of aliquots onto R2 no-sucrose plates. Wait until all the spotted drops are absorbed into the plates.
11. Incubate the plates at 30 °C for 16–18 h.
12. Flood the plates with 2 mL of a mixture of nalidixic acid and apramycin each at a concentration of 1 mg/mL (*see Note 19*).
13. Incubate the plates at 30 °C for additional 3–5 days until exconjugants appear, at which point exconjugants are picked and restreaked on ISP2-Apr⁺ plates and allowed to grow for 2 days.
14. Inoculate single colonies from the ISP2-Apr⁺ plates into 10 mL of MYG supplemented with 50 $\mu\text{g}/\text{mL}$ apramycin and grow the cultures at 30 °C for 2 days as seed cultures, of which 2.5 mL is subsequently inoculated to 250 mL of fresh MYG supplemented with 50 $\mu\text{g}/\text{mL}$ apramycin and grown for another 84 h.

3.7 Detection of Aureothin and Its Derivatives

1. Centrifuge the cultures at $3,220 \times g$ for 10 min to remove the cells.
2. Extract the supernatants with an equal volume of ethyl acetate and evaporate to dryness using rotary evaporator.
3. Perform LC–MS on an Agilent 1100 series LC/MSD XCT plus ion trap mass spectrometer with an Agilent SB-C18 reverse-phase column. HPLC parameters for detection of aureothin and its derivatives are as follows: solvent A, 1 % acetic acid in water; solvent B, acetonitrile; gradient, 10 % B for 5 min, to 100 % B in 10 min, maintain at 100 % B for 5 min, return to 10 % B in 10 min, and finally maintain at 10 % B for 7 min; flow rate 0.3 mL/min; detection by UV spectroscopy at 367 nm. Under such conditions, aureothin and its derivative are eluted at 17.8 min and 15.8 min, respectively. Mass spectra are acquired in ultra-scan mode using electrospray ionization (ESI) with positive polarity. The MS system is operated using a drying temperature of 350 °C, a nebulizer pressure of 35 psi, a drying gas flow of 8.5 L/min, and a capillary voltage of 4,500 V (*see Note 20*).

4 Notes

1. If a larger biochemical pathway needs to be assembled, increasing the length of the overlaps between the adjacent fragments is necessary. For example, to assemble a pathway with a size of ~25 kb, a longer overlap (e.g., 125 bp) could ensure high

assembly efficiency (>50 %), while low efficiency (10–20 %) is obtained if the length of the overlaps is only 50 bp [13].

2. *S. cerevisiae* is used as the assembly host, *E. coli* serves as the DNA enrichment host, and *S. lividans* is a widely used heterologous host for studying gene clusters from other *Streptomyces* species.
3. As shown in Fig. 2c, to ensure high assembly efficiency, overlaps of 400 bp between internal adjacent pathway fragments are generated. For example, the forward primer for amplifying the second pathway fragment can be located at ~400 bp upstream of the annealing position of the reverse primer for amplifying the first pathway fragment. Overlaps between other fragments are generated by adding tails to primers, thus they could not be very long. We encountered difficulties in amplifying the *S. cerevisiae* helper fragment, and in the end, the correct product was only obtained by using the pair of primers without any tails. As a result, the *S. cerevisiae* helper fragment only overlaps with the last pathway fragment and the adjacent helper fragment with 40 bp. Overlaps of 80 bp are generated between the *S. lividans* helper fragment and its neighbors.
4. Site-directed mutagenesis can be easily performed using DNA assembler. Unlike the labor-intensive and time-consuming procedures used in the conventional methods [26–28], DNA assembler only requires adding site-specific mutation(s) into the PCR primers used to generate pathway fragments. As an example, the active sites of the dehydratase domains (DH) of AurB are targeted (Fig. 2b). The motifs HXXXGXXXXP and DXXX(Q/H) were found to be conserved among the DH domains of polyketide synthases and the histidine and the aspartic acid were identified as the catalytic residues [29–31]. Based on this information, AurB DH H964A mutant and AurB DH H964A/D1131A double mutant will be generated. To confirm the necessity of gene(s), gene disruption can be carried out by two strategies: (1) a stop codon can be introduced into the early portion of the gene, resulting in in situ gene inactivation; (2) the complete gene can be omitted in pathway assembly by redesigning the overlaps. Refer to ref. 14 for more details.
5. The *hisG* and *delta2* sequences are not essential for assembling a pathway. As long as the 5' end of the first promoter and the 3' end of the last terminator contain overlaps (at least 50 bp) with the vector backbone, the pathway can be assembled on a plasmid. Similarly, any linearized *S. cerevisiae*–*E. coli* shuttle vector containing an *ura3* gene as a selection marker can be used as the vector backbone.
6. *S. cerevisiae* HZ848 is used as the host for DNA assembly. However, any *S. cerevisiae* strain with a nonfunctional *ura3* gene can be used as a host.

7. In order to verify the correctly assembled constructs through restriction digestion, a set of digestions consisting of one or two enzymes that cut the expected construct multiple times are chosen. Usually, two to three sets of digestions need to be set up in order to ensure the correct assembly. For a plasmid with a size of 15–20 kb, such as pRS416-zeaxanthin, find one or two enzymes which cut the DNA molecule 5–9 times. For a plasmid with a size of 20–30 kb, such as pZS-aur-2, find one or two enzymes which cut the DNA molecule 9–13 times. Try to avoid using enzyme digestion that will result in multiple fragments with similar sizes. Besides restriction digestion, the correctly assembled constructs can be confirmed by DNA sequencing.
8. In the construction of the first gene expression cassette and the last gene cassette, the *hisG* sequence and the *delta2* sequence are also included. Therefore, for these two reactions, four fragments are spliced together.
9. The fragment mixture can be maintained at -20°C for several months.
10. We encountered difficulties in amplifying the *S. cerevisiae* helper fragment, and in the end, the correct product was obtained by using the designated reagents and PCR program mentioned above.
11. 58°C is used as a standard annealing temperature. In some cases, especially for amplifying certain fragments from *Streptomyces*, fine-tuning annealing temperature is often necessary for obtaining the correct amplicons or improving PCR yields. Due to the high GC content of *Streptomyces* genome ($>70\%$), including 5 % DMSO in the reaction mixture will reduce the chance of forming secondary structures in general, resulting in better amplification efficiency. However, in some cases, we did encounter lower amplification efficiencies or even failures when DMSO was used. Generally, when difficulties are encountered to amplify a certain gene, a set of PCR conditions with various annealing temperatures and inclusion or exclusion of 5 % DMSO need to be tested.
12. Normally, the doubling time for a *S. cerevisiae* laboratory strain is approximately 2 h.
13. Unlike *E. coli*, yeast competent cells need to be freshly prepared each time.
14. For an efficient electroporation, a time constant of 5.0–5.2 ms should be obtained.
15. Assembly efficiency is defined as the percentage of the correct clones among the transformants appearing on the plate. Usually, ten colonies are picked and an average efficiency of 60–80 % can be obtained for assembly of the two target pathways.

16. *E. coli* strain BW25141 was used for plasmid enrichment and verification. However, any *E. coli* strain suitable for DNA cloning, such as DH5 α and JM109, can be used.
17. The number of obtained *E. coli* transformants could vary from a few to several thousands. This is mainly due to the low quality of the isolated yeast plasmids. However, as long as colonies appear, experiments can proceed.
18. WM6026 is an auxotrophic *E. coli* strain whose growth relies on exogenously supplemented DAP.
19. Nalidixic acid is used to kill *E. coli* after it donates the plasmid into *S. lividans*, and apramycin is used to select the successful *S. lividans* exconjugants.
20. Aureothin has a molecular weight of 398.4 and the aureothin derivative generated by the AurB DH H964A mutant and the AurB DH H964A/D1131A double mutant has a molecular weight of 414.4. The MS2 fragmentation patterns of the produced compounds are compared with that of authentic aureothin. The products generated by the AurB DH H964A mutant and the AurB DH H964A/D1131A mutant exhibit the expected +16 patterns in the MS and MS2 profiles.

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