

Supplementary Information

Rapid Characterization and Engineering of Natural Product Biosynthetic Pathways via DNA Assembler

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Materials and Reagents

Streptomyces lividans 66 and *Streptomyces thioluteus* were obtained from the Agricultural Research Service Culture Collection (Peoria, IL). Plasmids pAE4 and *E. coli* strain WM6026 were a gift from William Metcalf (University of Illinois, Urbana). Complete sequences of plasmids and details of strain and plasmid construction can be obtained by request from the authors. The plasmid pRS416 was purchased from New England Biolabs (Beverly, MA). FailsafeTM 2x premix buffer G was purchased from EPICENTRE Biotechnologies (Madison, WI), which was used as the reagent to amplify pathway fragments. Synthetic complete drop-out medium lacking uracil (SC-Ura) was used to select transformants containing the assembled pathways and *Saccharomyces cerevisiae* HZ848 (*MAT α* , *ade2-1*, Δ *ura3*, *his3-11, 15*, *trp1-1*, *leu2-3, 112*, and *can1-100*) was used as the host for DNA assembly.

DNA Manipulation and Yeast Transformation

S. thioluteus were grown in liquid MYG medium (4 g/L yeast extract, 10 g/L malt extract, and 4 g/L glucose) at 30 °C with constant shaking (250 rpm) for 2 days. The genomic DNA was isolated using the Wizard Genomic DNA isolation kit from Promega (Madison, WI). Pathway fragments were amplified from the genomic DNA of *S. thioluteus*, and the fosmid 79c containing the spectinabilin biosynthetic pathway.¹ The *S. cerevisiae* helper fragment was amplified from the plasmid pRS416, while the *E. coli* helper fragment and the *S. lividans* helper fragment were amplified from the *Streptomyces-E. coli* shuttle vector pAE4. Following electrophoresis, the PCR products were individually gel-purified from 0.7% agarose using Qiagen gel purification kit (Valencia, CA). 200-300 ng of each individual fragment was mixed and precipitated with ethanol. The resulting DNA pellet was air-dried and resuspended in 4 μ L Milli-Q double deionized water.

To construct the aureothin biosynthetic pathway and the intermediate plasmids carrying the partial spectinabilin biosynthetic pathway (**Fig. S5**), the concentrated mixture of DNA was electroporated into *S. cerevisiae* using the protocol reported previously.² To construct the full-length spectinabilin pathway, the three intermediate plasmids were subjected to *PacI* and *SspI* digestion and the released intermediate pathway fragments were combined with the master helper fragment (**Fig. S5**). After being concentrated, the mixture was transformed to *S. cerevisiae* using the lithium acetate/single stranded carrier DNA/polyethylene glycol (PEG) method.³

Restriction Digestion Analysis and Mutants Confirmation

Colonies were randomly picked to SC-Ura liquid media and grown for one day, after which the plasmids from yeast were isolated using Zymoprep II Yeast Plasmid Miniprep kit (Zymo Research, CA). Yeast plasmids were transformed to *E. coli* strain BW25141 and selected on Luria Broth (LB) agar plates supplemented with 50 μ g/mL apramycin. Colonies were inoculated into 5 mL of LB media supplemented with 50 μ g/mL apramycin, and plasmids were isolated from the liquid culture using the plasmid miniprep kit from Qiagen. Plasmids isolated from *E. coli* were then subjected to restriction digestion. Usually, one or two enzymes cutting the target molecule at multiple sites were chosen. The reaction mixtures were loaded to 0.7% agarose gels to check for the correct restriction digestion pattern by DNA electrophoresis. The mutation regions carried by the AurB DH H964 mutant, the AurB DH H964A/D1131A double mutant, the spectinabilin pathway mutant carrying the inactivated *spnK* (MutK), and the two spectinabilin knock-out mutants (Δ M and Δ M+ Δ L) were confirmed by sequencing.

Heterologous Expression in *S. lividans*

The verified clones were transformed to *E. coli* WM6026⁴ and selected on LB supplemented with 19 µg/mL 2,6-diaminopimelic acid and 50 µg/mL apramycin agar plates. These transformants were then used as the donors for conjugal transfer of the assembled plasmids to *S. lividans* 66 following the “high-throughput” protocol described previously with the exception that the entire *E. coli/S. lividans* mixture was spread on a R2 no-sucrose plate in 2 µL aliquots.⁵ After incubation at 30 °C for 16-20 hrs, plates were flooded with 2 mL of a mixture of 1 mg/mL each nalidixic acid and apramycin and incubated at 30 °C for an additional 3-5 days, at which point *S. lividans* exconjugants were picked and restreaked on ISP2 plates supplemented with 50 µg/mL apramycin and allowed to grow for 2 days. A single colony was inoculated into 10 mL MYG medium supplemented with 50 µg/mL apramycin and grown at 30 °C for 2 days as a seed culture, of which 2.5 mL was subsequently inoculated to 250 mL fresh MYG medium and grown for another 84 hrs.

LC-MS Analysis

Cultures were cleared of cells by centrifugation. The supernatants were extracted with an equal volume of ethyl acetate and concentrated 1000-fold before high performance liquid chromatography (HPLC) analysis. HPLC was performed on an Agilent 1100 series LC/MSD XCT plus ion trap mass spectrometer (Agilent, Palo Alto, CA) with an Agilent SB-C18 reverse-phase column. HPLC parameters for detection of aureothin, spectinabilin, and their derivatives were 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 spectinabilin are eluted at 17.8 min and 20.4 min, respectively. Mass spectra were acquired in ultra scan mode using electrospray ionization (ESI) with positive polarity. The MS system was operated using a drying temperature of 350 °C, a nebulizer pressure of 35 psi, a drying gas flow of 8.5 L min⁻¹, and a capillary voltage of 4500 V.

Figure S1. 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 were 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 were generated between the *S. lividans* helper fragment and its neighbors.

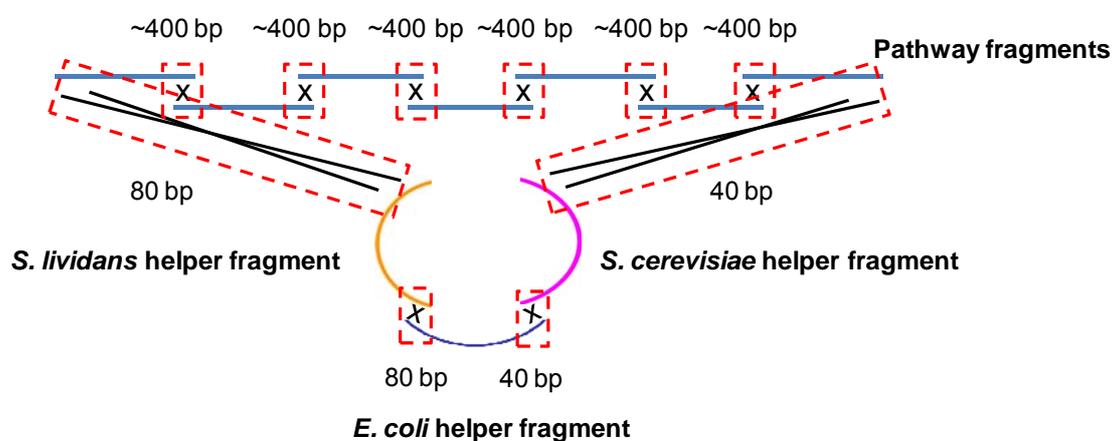


Figure S2. Vector maps of the assembled molecules carrying individual biosynthetic pathway. The *S. cerevisiae* helper fragment contains *CEN6* and *ARS H4* for replication, and *ura3* as a selection marker; the *E. coli* helper fragment contains *oriR6K* as an *ori* and *accIV* encoding for the apramycin resistance gene (it can be also used as the selection marker in *Streptomyces*); the *Streptomyces* helper fragment contains *oriT* for conjugal transfer *ori*, *PhiC31 attP* as the ϕ C31 recognition site, *int* encoding for the ϕ C31 integrase, and *tL3* as a terminator.

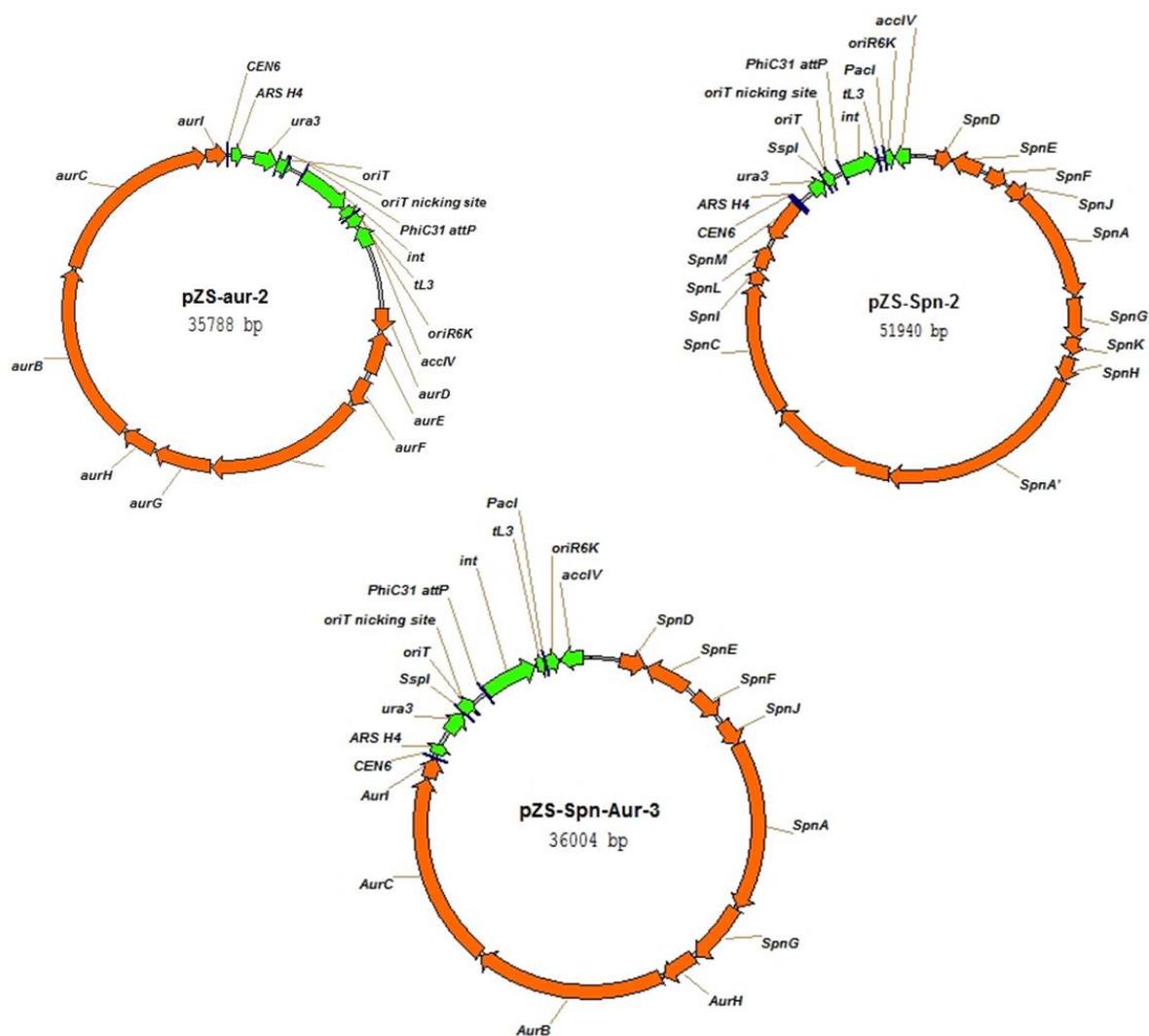


Figure S3. The HPLC analysis of the *S. lividans* strain as a negative control.

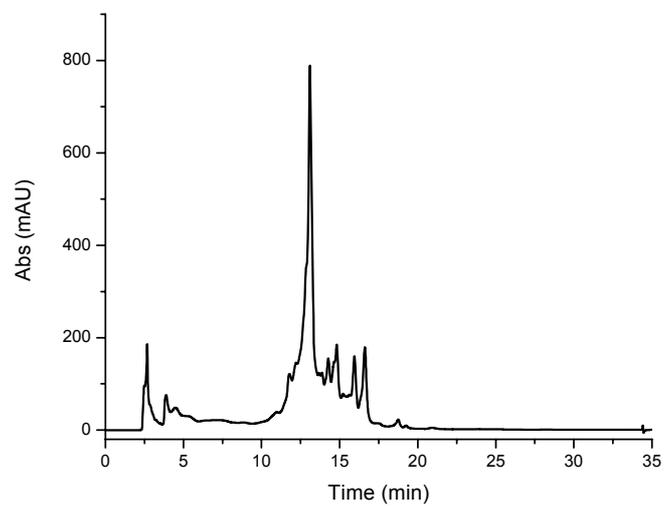


Figure S4. LC-MS analysis of the biosynthetic pathways producing aureothin and its derivatives. The products generated by the AurB DH H964A mutant and the AurB DH H964A/D1131A mutant exhibited the expected +16 patterns in MS and MS2 profiles.

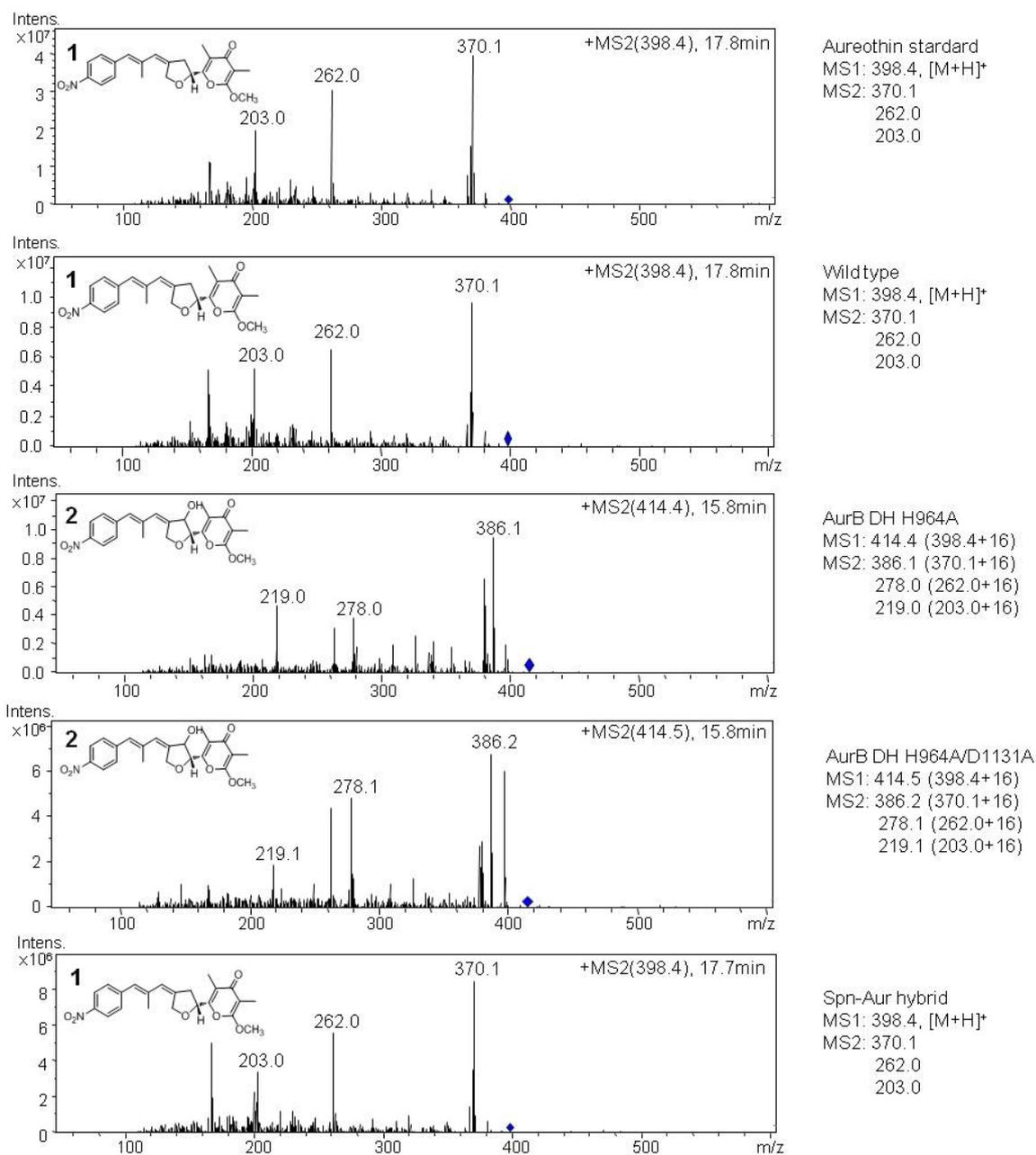


Figure S5. Comparison between the aureothin biosynthetic pathway from *S. thioluteus* and the spectinabilin biosynthetic pathways from *S. spectabilis* and *S. orinoci*.

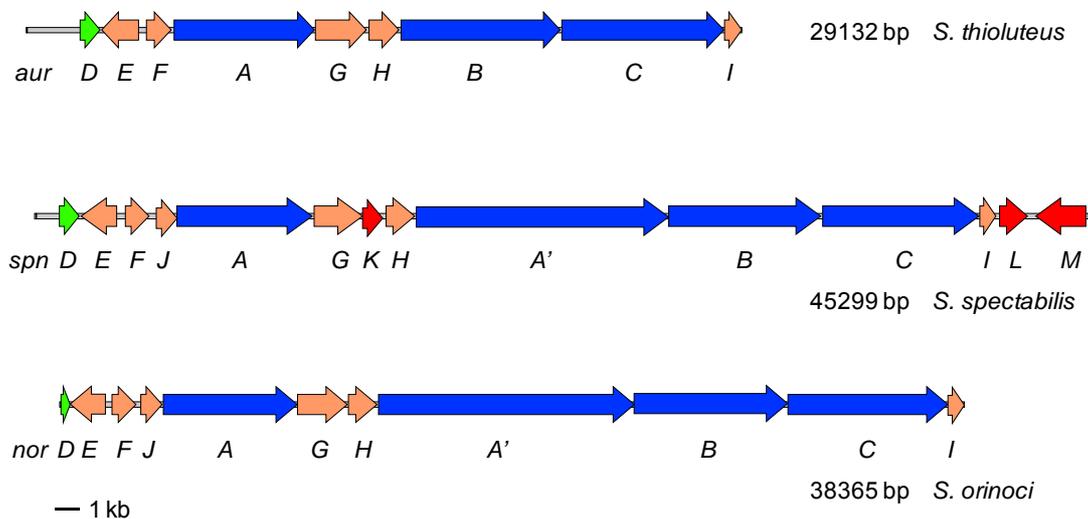


Figure S6. The two-step strategy for assembling the spectinabilin biosynthetic pathway. (a) The first step involved construction of three intermediate plasmids, separating the four PKSs into three plasmids. The first one contained *spnDEFJAGKH*, the second one contained the last 400 bp of *spnH*, the full-length *spnA'* and the first 400 bp of *spnB*, and the third intermediate plasmid contained *spnBCILM*. Two restriction sites, *SspI* and *PacI* were engineered at the appropriate positions of the intermediate plasmids. (b) Restriction digestion by *SspI* and *PacI* generated three intermediate fragments, which were co-transformed with a fragment obtained from restriction digestion of the master helper plasmids by *ApaLI* and *XhoI*. Here, we used the lithium acetate/single stranded carrier DNA/polyethylene glycol (PEG) method³ instead of the electroporation protocol² because we did not obtain colonies through electroporation. This might be due to the large sizes of the intermediate fragments (the largest one is about 20 kb). The master helper plasmid was constructed by co-transforming the three PCR-amplified helper fragments, which shared overlaps of 40-80 bp, into *S. cerevisiae*. E, Y, and S represent the corresponding *E. coli*, *S. cerevisiae*, and *Streptomyces* helper fragments.

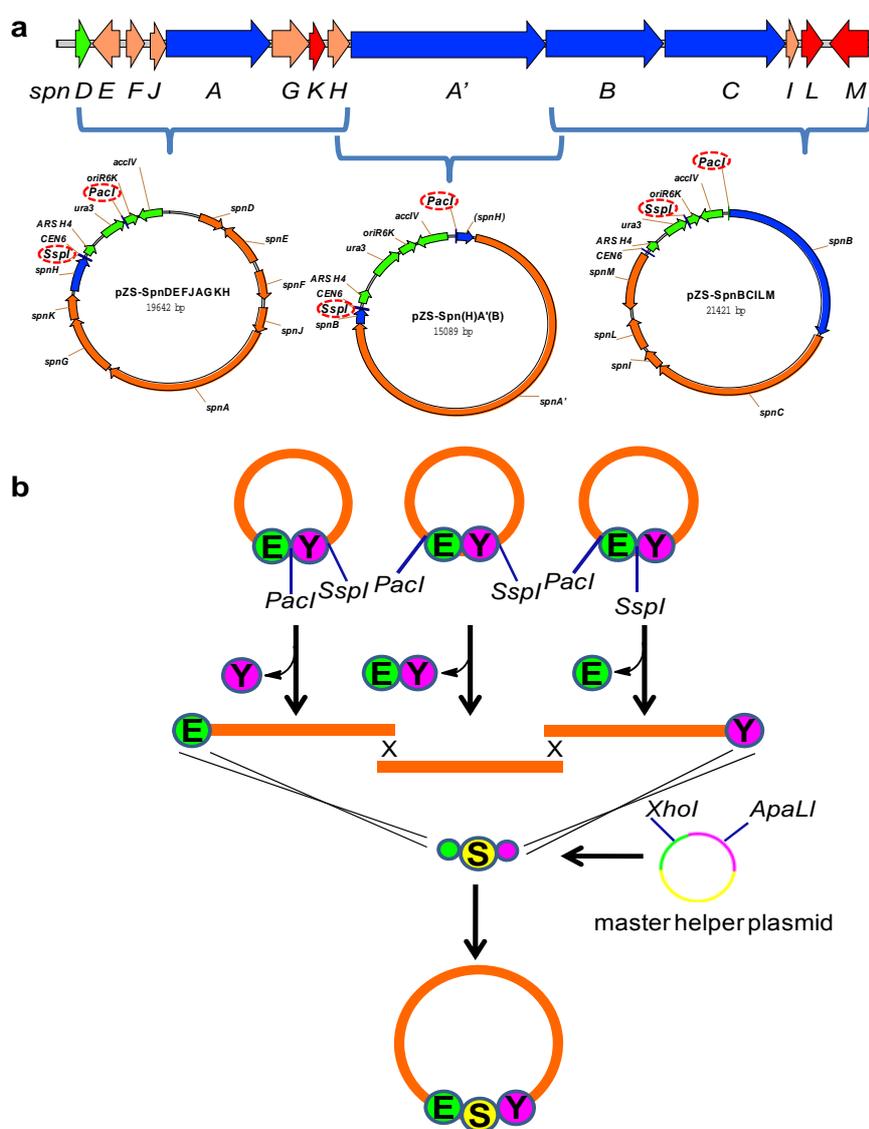


Figure S7. LC-MS analysis of the clones carrying the wild type and the mutant spectinabilin biosynthetic pathways.

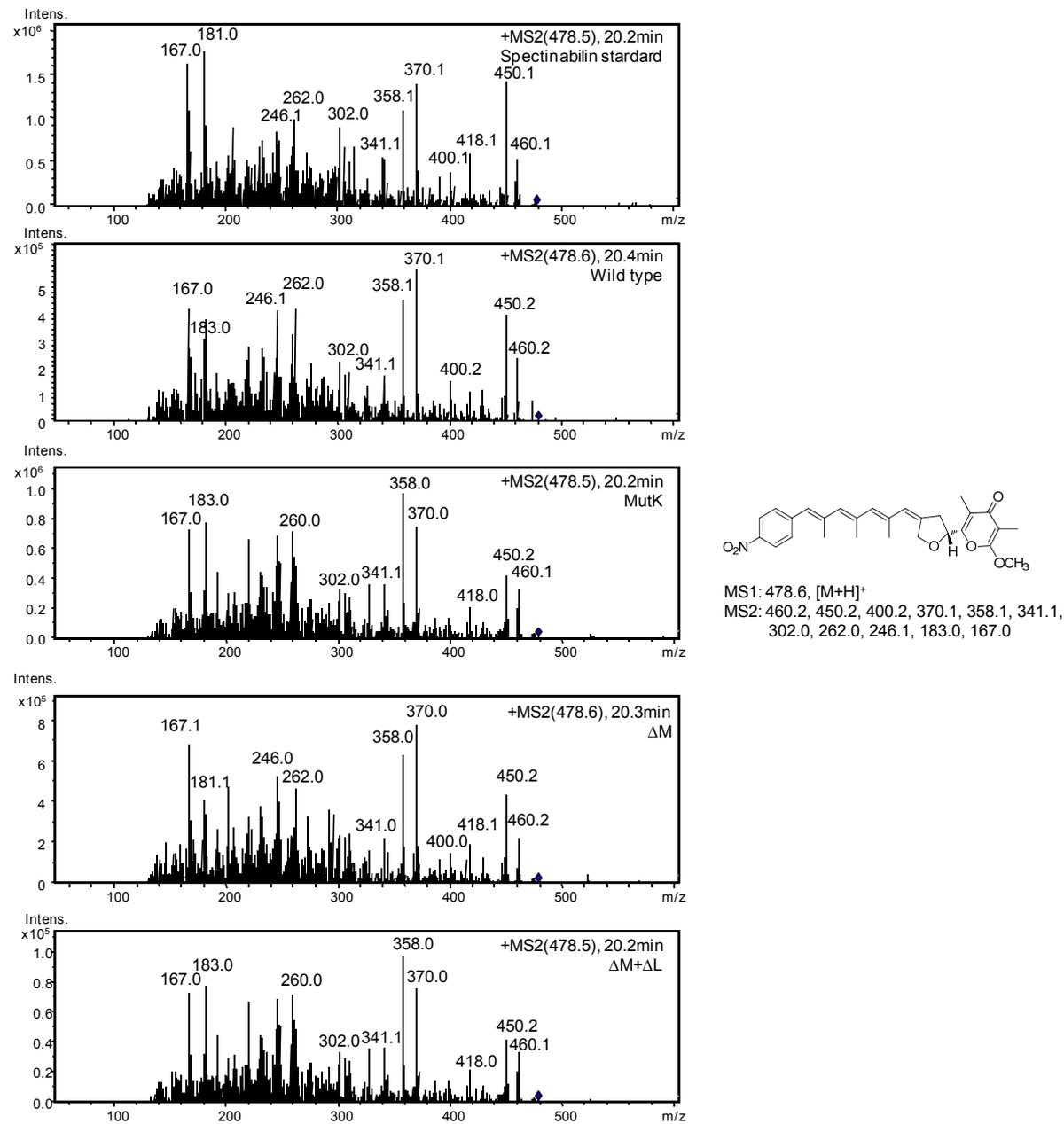


Figure S8. The N-terminal sequences of SpnA' and AurB, and SpnA' and SpnB were aligned using CLUSTALW, respectively.

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SpnA'      MTDDDRTLDYLKRLTAELGQTRERLRAVREAGREPIAVVSMACRFPGGVTSPEELWRLVG
AurB      MTNDAKTLEYLKRLTAELLETRERLRTAEAAADQEPVAVVSMGCRYPGGVSSPEDLWRLVT
          **: *  : ** : ***** : ***** : . . * . : ** : ***** . ** : ***** : ** : *****

SpnA'      SGTDGISALPTDRGWDTEGLYDPDPQRPGTTYAREGGFLADVTSFDAGLFGISPREALLM
AurB      DGTDAIAPFPADRGWNVDDLFDPDDRPGRTYTLEGGFVDGAAEFDADLFGISPREATAM
          . ** . * : . : ** : ***** : . : . : ** : ***** : ** * : ** : . . : . ** . ***** *

SpnA'      DPQQRLLLEVSWEALERAGLAPDALRGSRTGVFTGMMGQDYTDRKDSALTEYEGQLETGR
AurB      DPQQRLLLETAWETFERAGTDPGSLRGRPVGVFVGSLFVAGGSGVG-VAEGAEGYHMTGN
          ***** . : ** : ***** * . : ** * . * * . * : . . . * * ** .

SpnA'      -MTDDDRTLDYLKRLTAELGQTRERLRAVREAGREPIAVVSMACRFPGGVTSPEELWRLV
SpnB      MPAGDERFVEALRASLKEVETLRARNRELTAAREPIAVVAMGCRYPGGVRSPEDLWRLV
          : . : * : : * : * : * * * : * . ***** : * . ** : ***** * * : *****

SpnA'      GSGTDGISALPTDRGWDTEGLYDPDPQRPGTTYAREGGFLADVTSFDAGLFGISPREALL
SpnB      ETGTDAMSPFPADRGWTDALYDPDDRPGRTYTLEGGFVDGAGDFDADLFGISPREAAA
          : ** . : * . : ** : ***** : . ***** : ** * : ** : . . . ** . *****

SpnA'      MDPQQRLLLEVSWEALERAGLAPDALRGSRTGVFTGMMGQDYTDRKDSALTEYEGQLETG
SpnB      MDPQQRLLLETAWETFERAGVNPGALRGEPVGTFIGSVLMTVGNG-APATEGSEGHQVTG
          ***** . : ** : ***** : * . * * . . * . * * : . : * * * : ** : **
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References

1. Y. S. Choi, T. W. Johannes, M. Simurdiak, Z. Shao, H. Lu and H. Zhao, *Mol. Biosyst.*, **6**, 336-338.
2. Z. Shao, H. Zhao and H. Zhao, *Nucleic Acids Res.*, 2009, **37**, e16.
3. R. D. Gietz and R. A. Woods, *Methods Mol. Biol.*, 2006, **313**, 107-120.
4. R. D. Woodyer, Z. Shao, P. M. Thomas, N. L. Kelleher, J. A. Blodgett, W. W. Metcalf, W. A. van der Donk and H. Zhao, *Chem. Biol.*, 2006, **13**, 1171-1182.
5. A. Martinez, S. J. Kolvek, C. L. Yip, J. Hopke, K. A. Brown, I. A. MacNeil and M. S. Osburne, *Appl. Environ. Microbiol.*, 2004, **70**, 2452-2463.