Characterization of a Type III polyketide synthase from *Rhizobium etli*

Electronic Supplementary Information

1. Strains and materials

Reagents for polymerase chain reaction (PCR) -*Ex Taq*^{TM} DNA polymerase and T4 DNA ligase were purchased from Takara (Takara Corp., Japan). pGEM-T Easy was purchased from Promega (Madison, USA). A nickel-nitrilotriacetic acid (Ni-NTA) superflow column for purification was purchased from QIAGEN (Hilden, Germany). Restriction enzymes were obtained from New England Biolabs (MA, USA). pET28a expression vector was purchased from Novagen (Madison, WI). A plasmid isolation kit and oligonucleotide primers were obtained from Bioneer (Daejeon, South Korea). Electrophoresis reagents were from Bio-Rad, and all chemicals including Coenzyme A (CoA), malonyl-CoA, CoA esters of C_2-C_{18} fatty acids were from Sigma-Aldrich (St. Louis, Mo, USA). *R. etli* CFN42 was obtained from the Korean Agriculture Culture Collection (KACC10799; Suwon, South Korea). *E. coli* DH-5α and *E. coli* CodonPlus (DE3)-RIL were used as hosts for transformation of plasmid and expression, respectively. Both the *E. coli* strains were grown in Luria–Bertani medium supplemented with appropriate antibiotics at 37 °C.

2. Cloning, expression, and purification of RePKS

Multiple sequence alignment and secondary structure prediction of various type III PKSs is shown in **Figure S1.** RePKS-encoding open reading frame AAM55027.1 was amplified from *R. etli* DNA and initially cloned with an amino terminal hexahistidine affinity tag. For the production of His_6-tagged RePKS, *E. coli* BL21-CodonPlus (DE3)-RIL harboring pET28a-RePKS was grown at 37 °C in Luria–Bertani broth containing 50 μg mL^{-1} kanamycin and 30 μg mL^{-1} chloramphenicol. Once the OD600 reached 0.4–0.6, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the overexpression of RePKS. The induced culture was maintained at 16 °C for an additional 12 h. Cells were harvested by centrifugation, resuspended in lysis buffer composed of 20 mM Tris–HCl (pH 8.0) and 0.5 M NaCl, and disrupted by sonication. A crude cell-lysate was prepared by removing cell debris by centrifugation at 20,000 rpm at 4 °C for 30 min. RePKS was purified by using nickel–nitrilotriacetic acid (Ni–NTA) agarose (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The purified His_6-tagged protein was concentrated and desalted against buffer A [50 mM Tris–HCl (pH 8.0)] using a centrifugal filter (Millipore, Billerica, MA, USA).

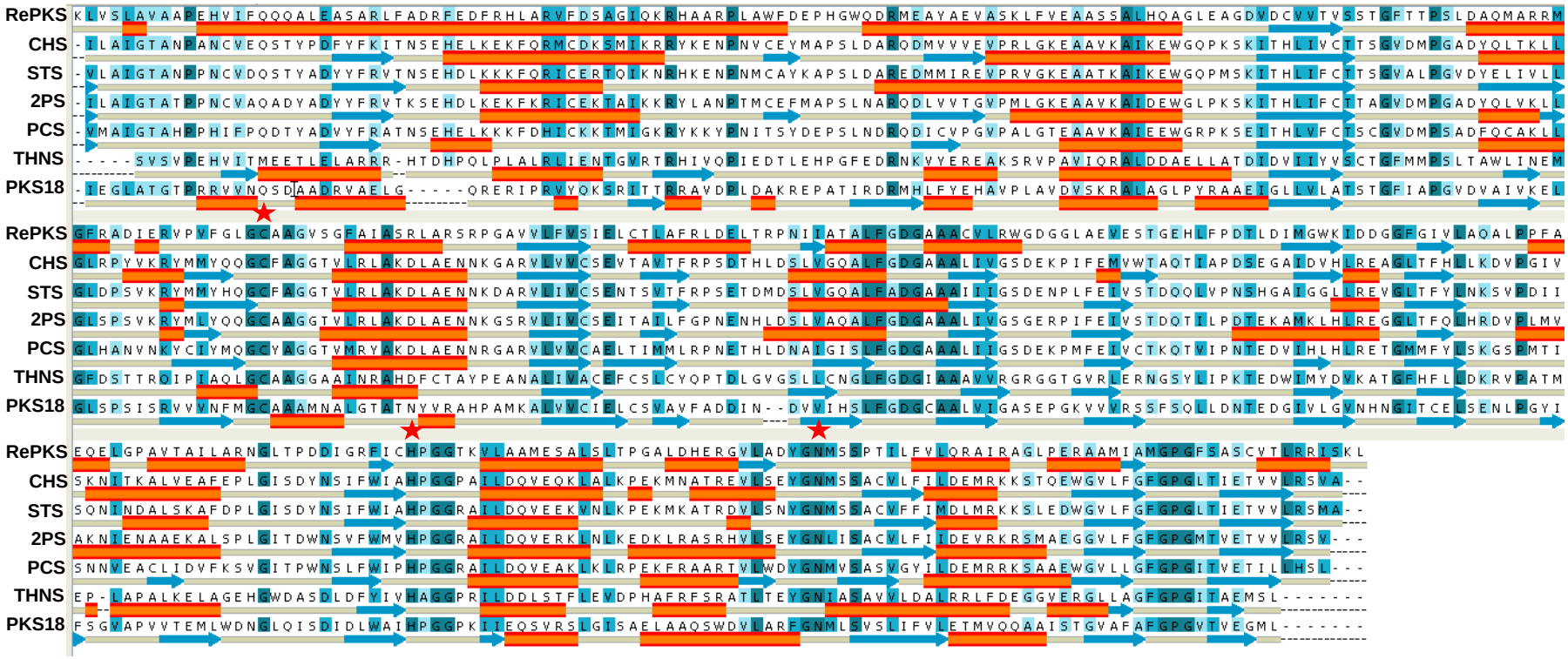

Figure S1 Multiple sequence alignment and secondary structure prediction of various type III PKSs. RePKS- type III PKS from *Rhizobium etli*; CHS- chalcone synthase from *Medicago sativa*; STS- stilbene synthase from *Pinus sylvestris*; 2PS- 2-pyrone synthase from *Gerbera hybrida*; PCS- pentaketide chromone synthase from *Aloe arborescens*; STCS- stilbene carboxylate synthase from *Marchantia polymorpha*; THNS-1,3,6,8-tetrahydroxynaphthalene synthase from *Streptomyces coelicolor* A3(2); ORAS- type III PKS from *Neurospora crassa*; PKS18- type III from *Mycobacterium tuberculosis*. Residues that constitute the catalytic triad are shown with a star.

3. Polyketide synthase assay and determination of kinetic parameters

The standard reaction mixture contained 100 µM malonyl CoA, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 40 µg RePKS in a total volume of 500 µl. After the mixture had been pre-incubated at 30 °C for 3 min, the reaction was initiated by adding malonyl CoA and was allowed to continue for 10 min. The reaction was stopped by adding 20 µl 4% TFA. Products were extracted with 600 µl ethyl acetate. The organic layer was dried by N_2 flushing, and the residual material was dissolved in 35 µl CH_3OH for HPLC analysis. Reverse-phase HPLC was conducted under the following conditions: ODS-80Ts (C18) column (4.6 × 150 mm; Tosoh), with a flow rate of 0.8 ml/min. Gradient elution was performed with H_2O and MeOH, both containing 0.1% trifluoroacetic acid: 0–5 min, 30% MeOH; 5–17 min, linear gradient from 30 to 60% MeOH; 17–25 min, 60% MeOH; 25–27 min, linear gradient from 60 to 70% MeOH. Elutions were monitored by a multichannel UV detector (MULTI 340, JASCO) at 290 and 330 nm; UV spectra (198–400 nm) were recorded every 0.4 s. On-line LC/ESI-MS spectra were measured with a Hewlett-Packard HPLC 1100 series (Wilmington, DE) coupled to a Thermo Finnigan LCQ DECA XP ion trap mass spectrometer (San Jose, CA, USA) fitted with an electrospray ionization source. HPLC separations were carried out under the same conditions as described above. The electrospray ionization capillary temperature and capillary voltage were 275 °C and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in both negative and positive mode over a mass range of *m/z* 150–400, at a range of one scan every 2 s.

To confirm the real starter substrate, ^{14}C malonyl-CoA was used in the assay. Reaction was performed by incubating RePKS (100 µg) with [2-^{14}C] malonyl-CoA in the presence and absence of acetyl-CoA. The preference of acetyl-CoA was determined by using 20 µM malonyl-CoA including 1.7 µM [2-^{14}C] malonyl-CoA in the presence and absence of 50 µM acetyl-CoA in the reaction mixture. The experiments were carried out in triplicate with 100 µg of purified enzyme in a final volume of 500 µL of 100 mM potassium phosphate buffer, pH 8.0. Incubations were carried out at 30 ^{o}C for 30 min as reported by Funa et al.[1] The reaction products were extracted and resolved on Silica gel 60 F254 TLC plates (Merck) in ethyl acetate/hexane/acetic acid (63:27:5, v/v/v). Resolved radiolabeled products were quantified using a Fuji FLA-5000 phosphor imager. K_m and V_{max} values were determined by non-linear regression analysis of data using GraphPad Prism 5 (GraphPad software, Inc., CA, USA) with varying concentrations of malonyl-CoA by following the procedures described by Zha et al.[2] The starter specificity of RePKS was examined by monitoring the incorporation of [^{14}C] malonyl-CoA into the condensation products.

Product from Malonyl-CoA [**1a**]. 6-(2-(2,4-Dihydroxy-6-methylphenyl)-2-oxoethyl)-4-hydroxy-2-pyrone [1aa]: retention time was 14.7 min on HPLC; UV, λ_{max} 283 nm. ESI-MS: m⁄z 277 $[M+H]^+$. HRMS (FAB, positive): found for $[C_{14}H_{13}O_6]^+$ 277.0698; calcd. 277.0701.

Product from hexanoyl-CoA [**2b**]. 4-Hydroxy-6-pentyl-2-pyrone [**3b**]: retention time was 14 min on HPLC; UV, λ_{max} 286 nm LC/APCIMS negative, MS 181$[M-H]^-$; MS/MS (precursor ion at *m/z* 181), 137$[M - CO_2-H]^-$. HRMS (FAB, negative): found for $[C_{10}H_{14}O_3]^-$181.0874; calcd. 181.0871.

Products from lauroyl-CoA [**2d**]. 4-Hydroxy-6-undecyl-2-pyrone [**3d**]: retention time was 20.4 min on HPLC; UV, λ_{max} 286 nm LC/APCIMS negative, MS 265.2[M-H]$^-$; MS/MS (precursor ion at *m/z* 265.2), 221.2[M - CO_2-H]$^-$. HRMS (FAB, negative): found for [$C_{16}H_{26}O_3$]$^-$ 265.2120; calcd. 265.2123. 4-Hydroxy-6-(2'oxotridecyl)-2-pyrone [**4d**]: retention time was 19.8 min on HPLC; UV, λmax 286 nm LC/APCIMS negative, MS 307.19[M-H]$^-$; MS/MS (precursor ion at *m/z* 307.19), 263.2[M - CO_2-H]$^-$, 125.01[$C_6H_5O_3$]$^-$. HRMS (FAB, negative): found for [$C_{18}H_{28}O_4$]$^-$307.2017; calcd. 307.2012.

Products from stearoyl-CoA [**2g**]. 6-heptadecyl-4-hydroxy-2-pyrone [**3g**]: retention time was 27.4 min on HPLC; UV, λ_{max} 286 nm LC/APCIMS negative, MS 349.2[M-H]$^-$; MS/MS (precursor ion at *m/z* 349.2), 305.2[M - CO_2-H]$^-$. HRMS (FAB, negative): found for [$C_{22}H_{38}O_3$]$^-$ 349.2280; calcd. 349.2276. 4-hydrocy-6-(2'-oxononadecyl)-2-pyrone [**4g**]: retention time was 22.4 min on HPLC; UV, λ_{max} 286 nm LC/APCIMS negative, MS 391.2[M-H]$^-$; MS/MS (precursor ion at *m/z* 349.2), 347.2[M - CO_2-H]$^-$, 125[$C_6H_5O_3$]$^-$. HRMS (FAB, negative): found for [$C_{24}H_{40}O_4$]$^-$391.2312; calcd. 391.2315.

4. Homology modeling and substrate docking

The three dimensional homology model of *R. etli* RePKS was generated using the Build Homology Models (MODELER) module in Discovery Studio 3.0 (DS 3.0, Accelrys Software Inc., San Diego, CA), with the crystal structure of THNS (1,3,6,8-tetrahydroxy-naphthalene synthase or RppA, 1UOM) used as a template. Comparative modeling generated the most probable structure of the query protein by alignment with template sequences, while satisfying spatial restraints and local molecular geometry. Sequence identity between the target and the template was found to be 31% according to BLAST parameters. The fitness of the models in their current 3D environment was evaluated by Profiles-3D Score/Verify Protein (MODELER) as implemented in DS 3.0. Discrete optimized protein energy scores in MODELER were also calculated to determine the quality of the protein structures. Profile-3D score was 216 against a maximum expected score of 232. The root mean square deviation (RMSD) between the models and the template was calculated by superimposing the model on the template crystal structure to assess the reliability of the models. The RMSD was 0.8 Å based on C-alpha atoms. The generated structure was improved by subsequent refinement of the loop conformations by assessing the compatibility of an amino acid sequence to known PDB structures using the Protein Health module in DS 3.0. The geometry of the loop regions was corrected using Refine Loop/MODELER. The modeled RePKS structure was submitted to PMDB with id: PM0077427.Malonyl CoA was docked into the CoA binding tunnel of the RePKS model using CDOCKER, a molecular dynamics simulated-annealing-based algorithm module in DS 3.0. The substrate orientation which gave the lowest interaction energy was chosen for subsequent rounds of docking.[3]

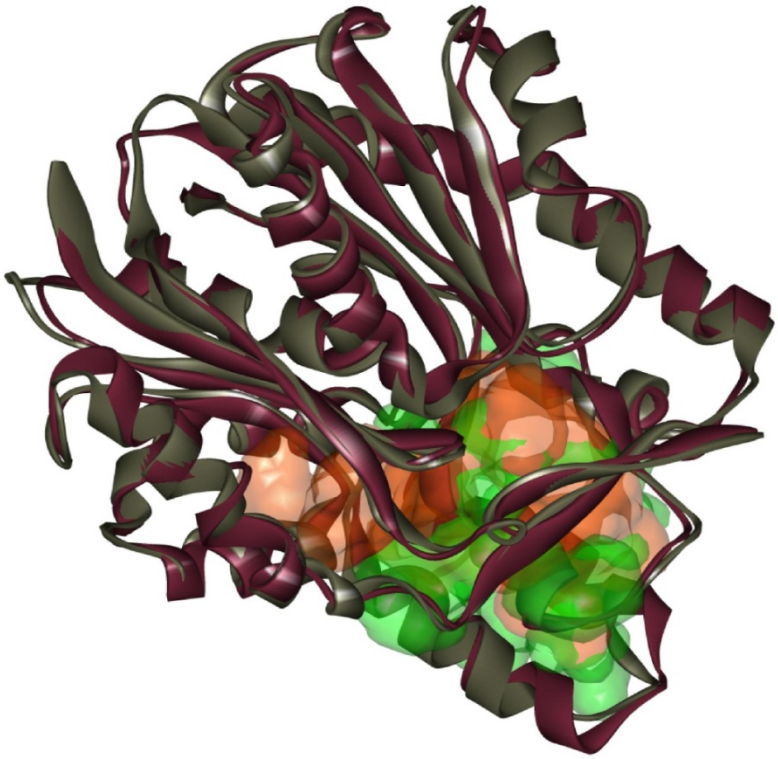

Figure S2 Superposition of the three-dimensional structures of *R. etli* PKS (RePKS; maroon) and the bacterial type III PKS (*S. coelicolor* THNS; grey) shown along with their cavities. Active site cavity of THNS is shown in orange and that of RePKS is represented in green color. The cavity volume of RePKS is larger (756.8Å^3) than that of THNS (622.0 Å^3).

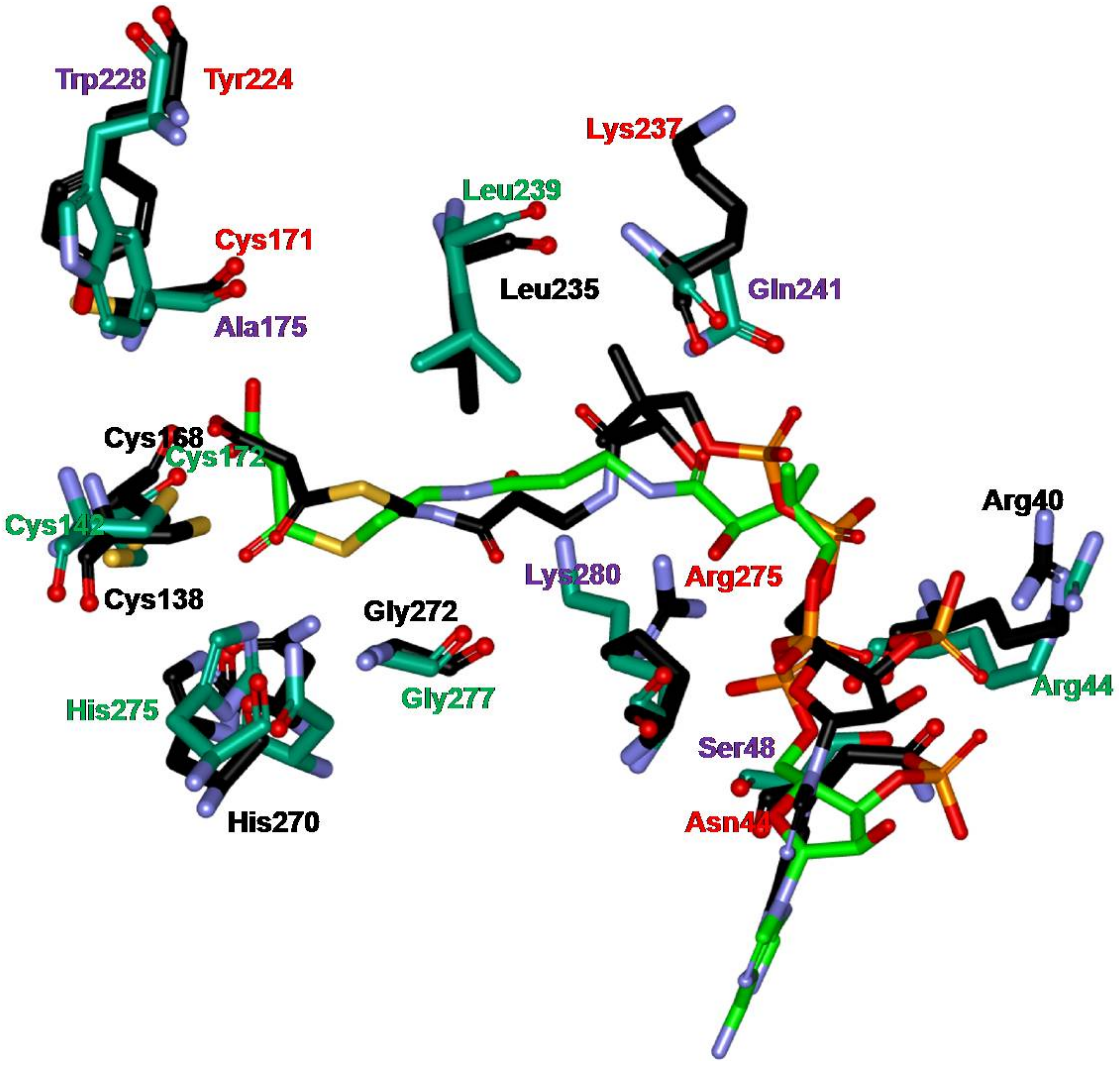

Figure S3 Superimposition of the RePKS + malonyl CoA complex and the 1U0M + malonyl CoA complex. Amino acid residues of RePKS and docked malonyl CoA are shown in green color. Amino acid residues of 1U0M and docked malonyl CoA are shown in black color. Amino acid residues that are different in 1U0M and RePKS are labeled in red and purple color, respectively.

5. Site-directed mutagenesis of RePKS

Site-directed mutagenesis was carried out using a QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The recombinant plasmid pET28-*Repks* containing the WT *pks* gene was used as the DNA template. Plasmids containing the correct mutant genes were then used to transform *E. coli* BL21 (DE3, codon plus), and colonies selected by kanamycin and chloramphenicol resistance were used for protein expression. The purified mutant proteins are shown in **Figure S4**.

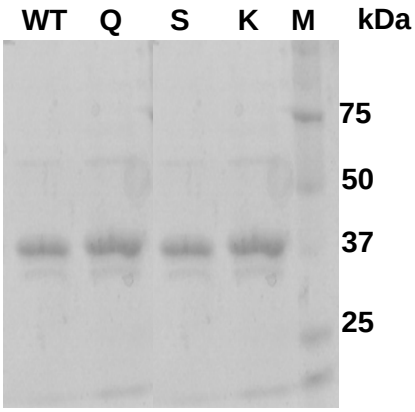

Figure S4 SDS-PAGE analysis of wild-type RePKS (WT) and alanine mutants, with molecular weight in kilodaltons. Lane M shows the protein markers. Lanes – Q, S and K corresponds to Q241A, S48A and K280A purified mutant enzymes of RePKS with a molecular weight of *ca.*37 kDa.

Table S1. Kinetic parameters determined for the RePKS wild-type and mutants

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
WT	4.3 ± 0.5	22.5 ± 3.5	5230 ± 185
A175C	443 ± 33	28.5 ± 4.5	64.7 ± 4.1
W228A	544 ± 44	41.3 ± 7.5	76.4 ± 6.3
Q241A	197 ± 16	14.3 ± 1.4	72.6 ± 1.3
K280A	171 ± 12	14.0 ± 2.1	83.6 ± 4.3
S48AA	144 ± 9	14.6 ± 1.8	101 ± 6

Kinetic parameters of RePKSs were shown for malonyl CoA.

Notes and references

1. N. Funa, T. Awakawa and S. Horinouchi, *J Biol Chem*, 2007, **282**, 14476-14481.
2. W. Zha, S. B. Rubin-Pitel and H. Zhao, *J Biol Chem*, 2006, **281**, 32036-32047.
3. M. Tiwari and J. K. Lee, *J Mol Graphics Modelling*, 2010, **28**, 707-713.