Supporting information for

Role of a Conserved Arginine Residue in Linkers between the Ketosynthase and Acyltransferase Domains of Multimodular Polyketide Synthases

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EXPERIMENTAL PROCEDURES

Reagents and Chemicals. DL-2-Methyl-^{14}C]-methylmalonyl-CoA was from American Radiolabeled Chemicals. [^{14}C](2*S*,3*R*)-2-Methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester (**1**) was prepared by custom synthesis by Amersham Pharmacia. Unlabeled diketide **1** was prepared as described previously (Cane and Yang, 1987). All other chemicals were from Sigma-Aldrich or BioRad. Thin-layer chromatography (TLC) plates were from J. T. Baker. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels (4-20%) were from BioRad. Ni-NTA affinity resin was from Qiagen. HiTrap-Q anion exchange column was from Amersham Pharmacia.

Expression and Purification of M3+TE. Plasmid pRSG34 (Gokhale et al., 1999) was introduced via transformation in an engineered *Escherichia coli* strain, BAP1 (Pfeifer et al., 2001), to ensure complete pantetheinylation of the ACP domain of M3+TE. Cells were grown in LB medium at 37 °C to an OD_{600} = 0.6 before the cultures were cooled to 18 °C and induced with 0.25 mM isopropyl-β-D-galactopyranoside (IPTG) for 16 h. The cells were harvested by centrifugation (4500*g*, 5 min) and resuspended in lysis/wash buffer (50 mM phosphate, pH 7.6, 300 mM NaCl, 10 mM imidazole). Cells were lysed with sonication (8 × 30 sec) and cellular debris was removed by centrifugation (17000*g*, 30 min). Nickel-NTA agarose resin was added directly to the supernatant (5 mL of resin/(L of culture)) and mixed for 1 h at 4 °C. This resin was poured into a fritted column, washed with 10 resin volumes of lysis/wash buffer, and eluted with 2 resin volumes of elution buffer (150 mM phosphate, pH 7.6, 50 mM NaCl, 150 mM imidazole). The eluted protein was applied to a HiTrap-Q anion exchange column and eluted at approximately 370 mM NaCl. M3+TE fractions were pooled, concentrated and the buffer was exchanged into stock buffer (50 mM phosphate, pH 7.6, 8% glycerol) to store the protein at -80°C.

Plasmid Construction, Expression and Purification of M3+TE Linker Mutants. Mutations were introduced into pRSG34 by using a method similar to the QuickChange Site-Directed Mutagenesis Kit

(Stratagene). The resulting plasmids were introduced via transformation in *E. coli* BAP1 to express the mutants in its holo form. The proteins were purified as described above.

Triketide Lactone Formation Assay. The overall catalytic sequence that yields the triketide lactone product is shown in Figure 1. Each time point was set up in a reaction volume of 10 μL. M3+TE or the linker mutants (10 μM) were incubated with 5 mM **1** and 200 μM DL-[2-methyl-^{14}C]-methylmalonyl-CoA in 100 mM phosphate buffer, pH 7.2, containing 2.5 mM tris(2-carboxyethyl)phosphine (TCEP) at 23 °C or 30 °C. At each time point (10 min, 20 min, or 30 min), the reaction was quenched by adding 10 μL of 1 M potassium hydroxide. Hydrochloric acid (10 μL of 1.5 M) was then added, and the mixture was dried in a Speed-vac for 2 h. The pellet was resuspended in 10 μL of ethyl acetate and spotted onto a TLC plate. A 3:2 mixture of ethyl acetate/hexane was used for TLC, and the radiolabeled products were then visualized and quantified using a phosphorimager (Packard InstantImager).

KS Acylation Assay. The reaction of interest is shown in step (1) of Figure 1. Each time point was set up in a reaction volume of 10 μL. M3+TE or the linker mutants (10 μM) were incubated with radiolabeled **1** (5 mM) in 100 mM phosphate, pH 7.2, 2.5 mM TCEP at 23 °C or 30 °C. At each time point (1 min, 2 min, or 4 min), the reaction was quenched with Laemmli Sample Buffer lacking any reducing agents. Samples were then resolved on a 4–20% SDS-PAGE gradient gel and visualized with Coomassie blue stain. The gel was dried using a BioRad gel-drying system and analyzed using a phosphorimager (Packard InstantImager).

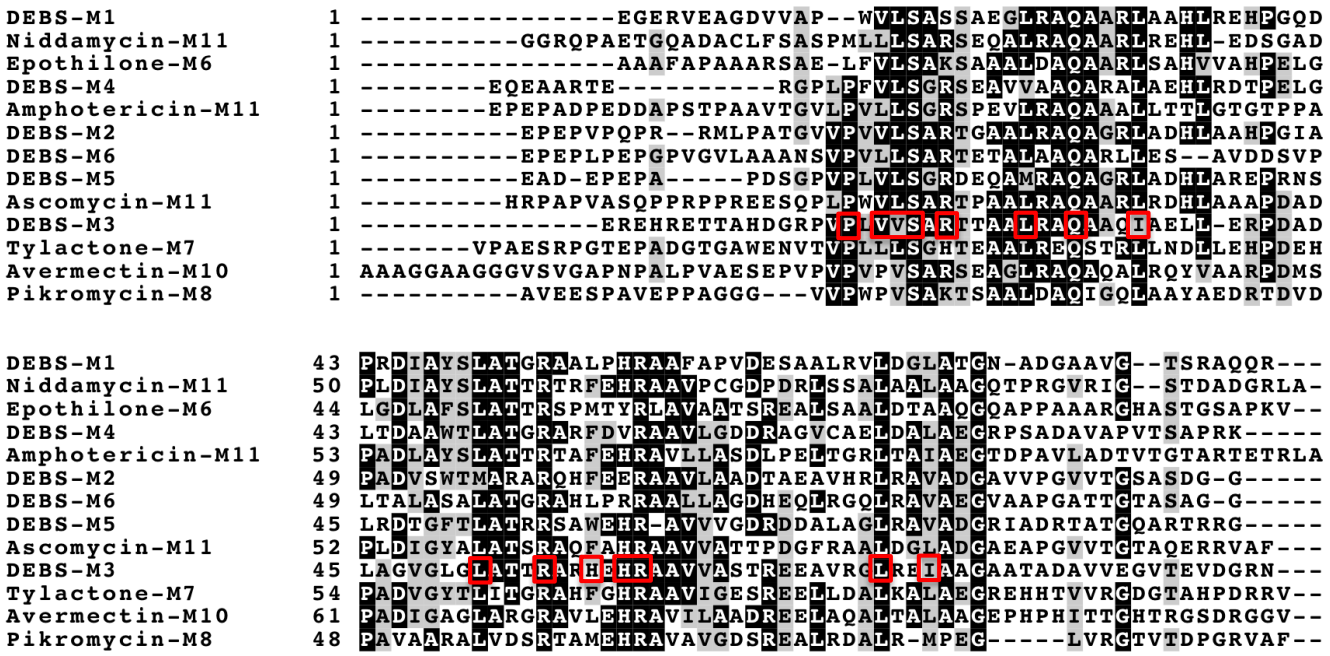
ACP Transacylation Assay. Each time point was set up in a reaction volume of 10 μL. M3+TE or the R513A mutant (1.25 μM) was incubated with 25 μM DEBS holo-ACP3 and 200 μM DL-[2-methyl-^{14}C]-methylmalonyl-CoA in 100 mM phosphate, pH 7.2, 2.5 mM TCEP at 23 °C. At 30 s, samples were quenched with Laemmli Sample Buffer lacking any reducing agents. Samples were then resolved

on a 4–20% SDS-PAGE gradient gel and visualized with Coomassie blue stain. The gel was dried using a BioRad gel-drying system and analyzed using a phosphorimager (Packard InstantImager).

Chain Translocation Assay. The reaction of interest is shown in Supplemental Figure 4A. For practical reasons, it is easier to assay back-translocation (i.e. the microscopic reverse of reaction 1), in which the diketide chain is transferred from the downstream KS to the upstream ACP. To do so, each time point was set up in a reaction volume of 10 μL. M3+TE or the linker mutants (38.5 μM) were incubated with radiolabeled **1** (1.15 mM) for 1 h at 23 °C in buffer A (100 mM phosphate, pH 7.2, 3.85 mM TCEP). Subsequent kinetic measurements were performed on ice and all components were prechilled. To measure the rate of chain translocation, DEBS holo-ACP2 was then added to radiolabeled M3+TE or the mutants such that the final concentration of each protein was 25 μM in buffer B (100 mM phosphate, pH 7.2, 2.5 mM TCEP, 12.5% v/v glycerol). At 30 sec, samples were quenched with Laemmli Sample Buffer lacking any reducing agents. The proteins were resolved on a 4–20% SDS-PAGE gradient gel and visualized with Coomassie blue stain. The gel was dried using a BioRad gel-drying system and analyzed using a phosphorimager (Packard InstantImager).

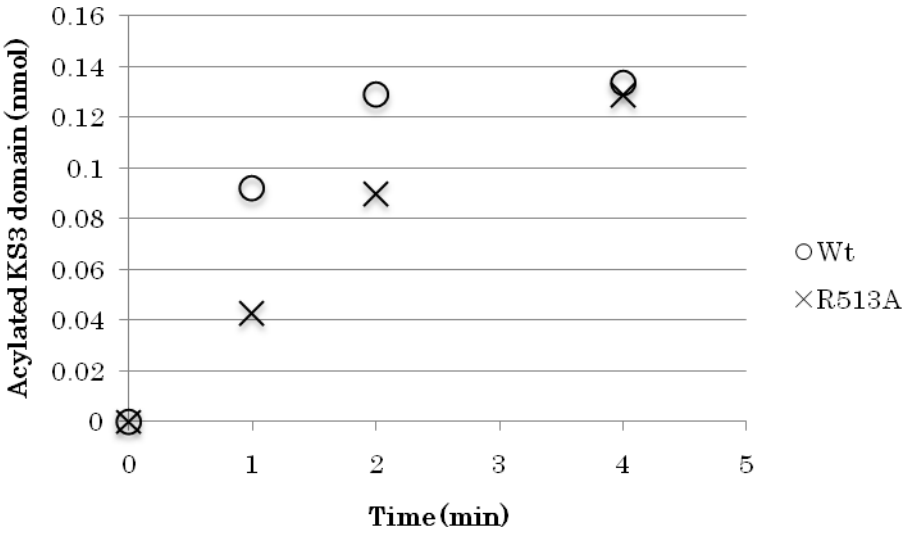
Limited proteolysis. Each time point was set up in a reaction volume of 5 μL. Purified M3+TE and the R513A mutant (1 mg/mL in 100 mM phosphate, pH 7.2, 2.5 mM TCEP) was digested with 1 μg/mL trypsin (T6567, Sigma-Aldrich) at 30 °C and quenched after 4 min, 16 min, and 64 min by addition of Laemmli Sample Buffer containing β-mercaptoethanol. The resulting products were analyzed on a 4–20% SDS-PAGE gradient gel. The proteolyzed products specific to R513A mutant (fragments between 50-60 kDa and 90-100 kDa) were characterized by N-terminal sequencing (automated Edman degradation).

FIGURES

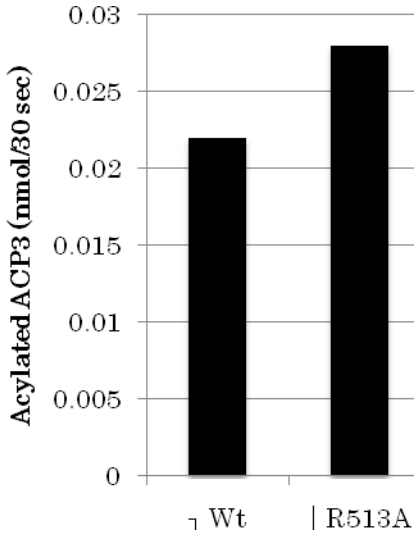


Supplemental Figure 1. Multiple sequence alignment of KS-AT linkers. KS-AT linker sequences from DEBS module 1 to 6 were aligned with homologs from other multimodular PKSs using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The alignment was produced using BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Based on the resulting alignment, Pro-473, Val-475, Val-476, Ser-477, Arg-479, Leu-484, Gln-487, Ile-491, Leu-509, Arg-513, His-516, His-518, Arg-519, Leu-534, and Ile-537 (numbering based on PDB ID: 2QO3 and shown in red rectangle) were selected as conserved residues.

(A)

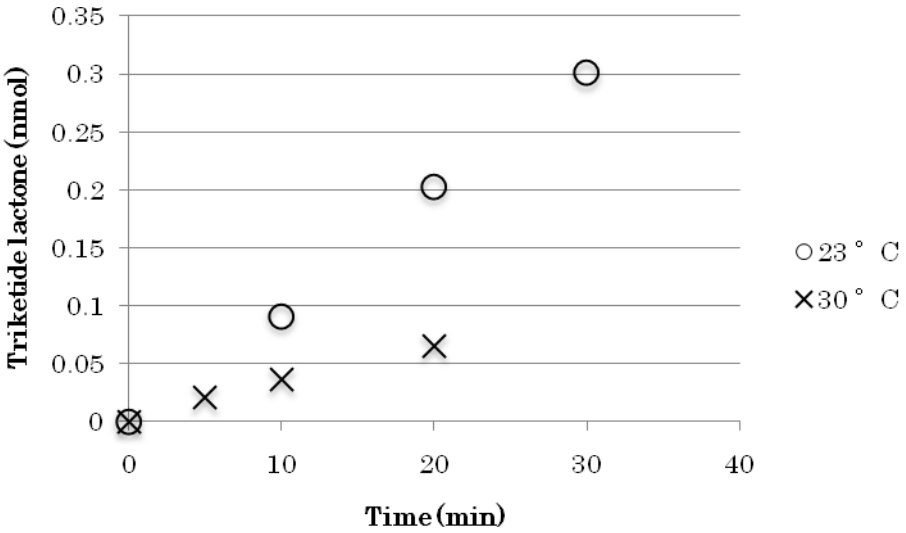


(B)

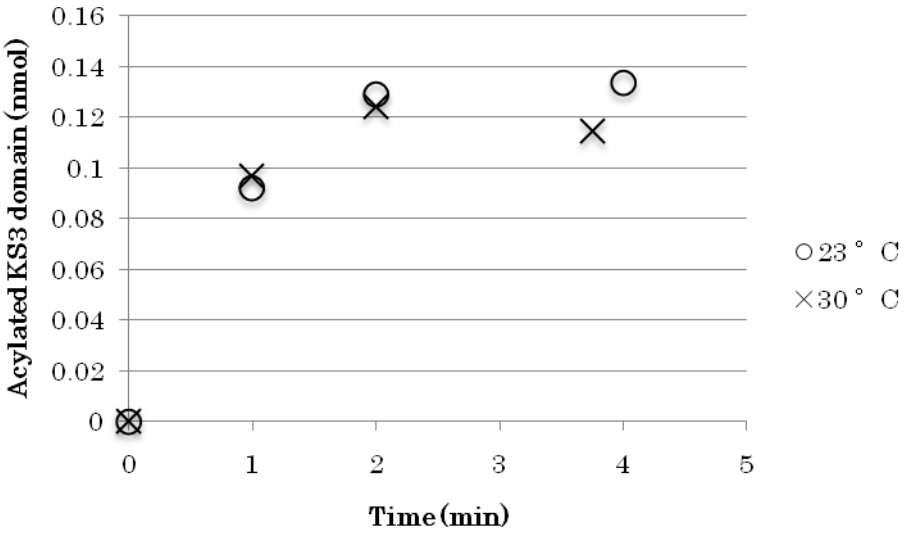


Suplemental Figure 2. (A) KS acylation rates of M3+TE and the R513A mutant. M3+TE or the R513A mutant (10 μM) was incubated with radiolabeled **1** (5 mM) at 23 °C. At each time point (1 min, 2 min, or 4 min), 10 μL of the reaction mixture was quenched and was then resolved on a 4–20% SDS-PAGE gradient gel. The gel was dried and analyzed using a phosphorimager. Open circles; M3+TE, crosses; R513A mutant. (B) Transacylation rates of the stand-alone ACP3 by M3+TE and the R513A mutant. M3+TE or the R513A mutant (1.25 μM) was incubated with 25 μM DEBS holo-ACP3 and 200 μM $_{DL}$-[2-methyl-^{14}C]-methylmalonyl-CoA at 23 °C. At 30 sec, 10 μL of the samples were quenched and then resolved on a 4–20% SDS-PAGE. The gel was dried and analyzed using a phosphorimager.

(A)

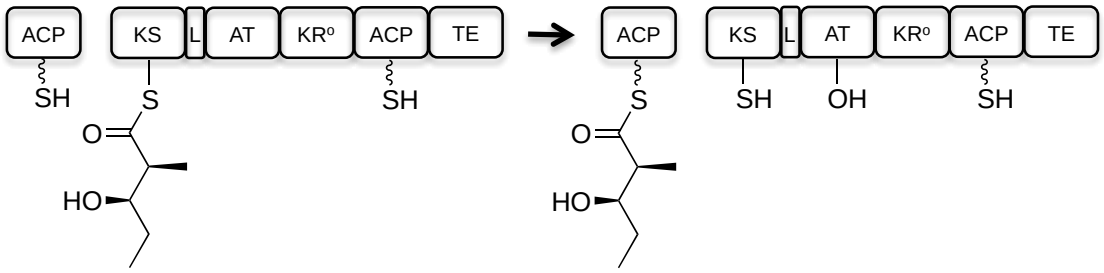


(B)

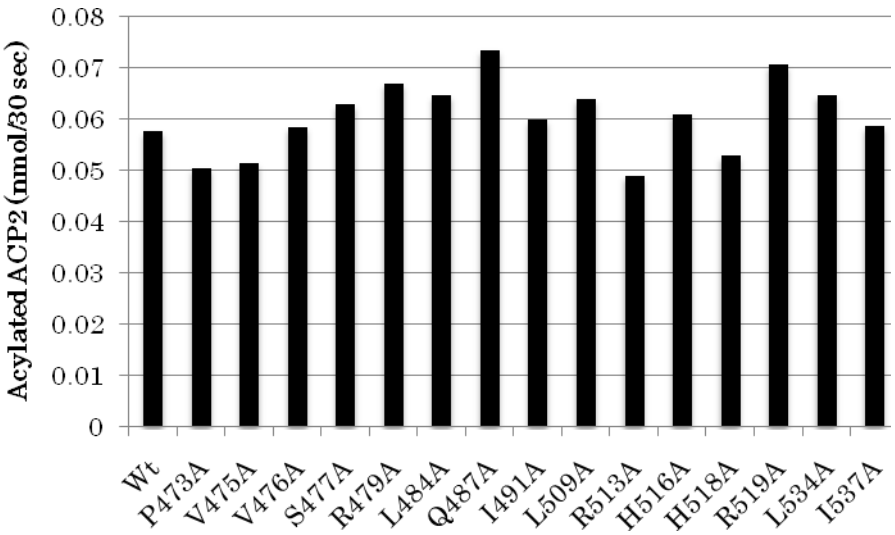


Supplemental Figure 3. (A) Triketide lactone formation rates catalyzed by M3+TE. M3+TE (10 μM) were incubated with 5 mM **1** and 200 μM $_{DL}$-[2-methyl-^{14}C]-methylmalonyl-CoA at 23 °C or 30 °C. At each time point (10 min, 20 min, or 30 min), 10 μL of the reaction mixture was quenched and the radiolabeled products were then visualized and quantified using a phosphorimager. (B) KS acylation rates of M3+TE. M3+TE (10 μM) was incubated with radiolabeled **1** (5 mM) at 23 °C or 30 °C. At each time point (1 min, 2 min, or 4 min), 10 μL of the reaction mixture was quenched and then resolved on a 4–20% SDS-PAGE. The gel was dried and analyzed using a phosphorimager. Open circles; 23 ℃, crosses; 30 ℃.

(A)



(B)



Supplemental Figure 4. Chain transfer rates of M3+TE and its KS-AT linker mutants. (A) Back-translocation of the polyketide chain by M3+TE. The module consists of a KS, a KS-AT linker (L), an AT, an inactive KR (KR°), and an ACP domain. In the presence of the upstream ACP2 and the absence of methylmalonyl-CoA, the KS-bound diketide, **1** can be transferred to ACP2. The phosphopantetheine prosthetic arm is shown as a wavy line. (B) The KS domains of M3+TE or the linker mutants were acylated with radiolabeled **1**. Subsequent kinetic measurements were performed on ice. To measure the rates of chain transfer, DEBS holo-ACP2 (25 μM) was added to radiolabeled M3+TE or the mutants (25 uM). At 30 sec, 10 μL of the samples were quenched and then resolved on a 4–20% SDS-PAGE. The gel was dried and analyzed using a phosphorimager.