

Supplementary Information

**Broadening substrate specificity of a chain-extending ketosynthase
through a single active-site mutation**

Annabel C. Murphy, Hui Hong, Steve Vance, R. William Broadhurst and Peter F. Leadlay

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General Analytical Procedures

HPLC-MS analysis was performed using an HPLC (Hewlett Packard, Agilent Technologies 1100 series) coupled to a Finnigan MAT LCQ mass spectrometer fitted with an electrospray ionization (ESI) source. The HPLC was fitted with a Jupiter 5 μ C4 column (2.0 x 250 mm, Phenomenex). A solvent system of CH₃CN and H₂O, both containing 0.1% trifluoroacetic acid, was used. Samples were eluted at a flow rate 0.3 mL min⁻¹ with a linear gradient of 5 to 45% CH₃CN over 5 min, followed by 45 to 55% CH₃CN over 13 min, then 55 to 95% CH₃CN over 2 min and finally 95% CH₃CN over 4 min. The mass spectrometer was run in positive ionization mode, scanning from m/z 200 to 2000, and the collision energy was set to 35%. HPLC-MS data were processed using Xcalibur (version 1.1, Thermo Finnigan) and deconvolution of the various charge states observed was carried out using Promass 2.8 (Novatia).

DNA sequencing was carried out by the DNA Sequencing Facility in the Department of Biochemistry, University of Cambridge. High resolution mass spectroscopy was carried out using positive ion electrospray ionization on a Thermo Fisher Orbitrap Mass Spectrometer.

Bacterial strains and culture conditions

E. coli strains (DH10B and BL21 DE3) were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or on LB agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) at 37 °C with kanamycin selection (50 μ g mL⁻¹).

Materials, DNA isolation and manipulation

Oligonucleotides used in this work are summarised in Table S1. Restriction endonucleases (Fast Digest) and Pfu DNA polymerase were purchased from Thermo Scientific, shrimp alkaline phosphatase was purchased from USB, T4 DNA ligase was purchased from New England Biolabs. Chemicals were from Sigma-Aldrich. Plasmid DNA was isolated from an overnight culture using the Plasmid Mini Kit I (Omega BioTek). Purification of DNA fragments from agarose gels was performed using the Anachem Gel Recovery Kit, then concentrated using the Zymo DNA Clean and Concentrator kit. PCR amplifications were carried out using a Mastercycler (Eppendorf) and the Phusion High-Fidelity PCR Master Mix (GC buffer) from New England Biolabs, with a final concentration of DMSO of 3-4%.

A C-terminal His-tag EryKS3AT3 was generated by amplification using the primers given in Table S1. The PCR product was digested with NdeI and EcoRI, purified by agarose gel, extracted and concentrated. This fragment was then ligated into pET29b that had been previously digested with the same restriction enzymes and shrimp alkaline phosphatase, purified by agarose gel, extracted and concentrated. The same protocol was used to clone EryACP3 into pET28a to generate EryACP3 with an N-terminal His-tag. The MlsAT9 gene was cloned into a pET28a(TEV) (N-terminal sequence: MGSSHHHHHSSGENLYFQG) using the primers given in Table S1 by one step, isothermal Gibson Assembly.^[1]

For general DNA manipulations, *E. coli* strain DH10B was used, while for protein expression *E. coli* strain BL21 DE3 was used. Chemically competent cells were generated using calcium chloride and then transformed with the plasmids described above, and selected on LB agar plates containing kanamycin (50 µg mL⁻¹).

Site-directed mutagenesis was carried out using the Quikchange method (Stratagene) with inclusion of DMSO at 6% and the primers given in Table S1.

Model generation

The amino acid sequence of the 16 MLS KS domains were aligned with the KS domain of DEBS module 3 and a sequence identity of 63% was found (excluding the DEBS N-terminal docking domain). An *in silico* model of the KS from MlsA2 was generated using Phyre2 and iTASSER.^[2,3] Beyond minor differences in side-chain placement, the programs generated very similar models of the active site and the Phyre2 model was then compared with the EryKS3AT3 didomain crystal structure.

Protein expression and purification

Expression of EryKS3AT3 and all mutant variants was carried out in 150 mL LB in 500 mL Ultra Yield flasks (Thompson) with kanamycin selection and, after incubation at 37 °C until an OD₅₀₀ of 0.6, the culture was cooled to 22 °C and induced with IPTG (0.2 mM) for 16 h. Purification was then carried out by incubating the lysed cell supernatant (10 mM imidazole, 50 mM sodium phosphate pH 7.2, 300 mM NaCl) with Ni-NTA resin at 4 °C for 1 hour, washing with 10 column volumes of the above loading buffer, then eluting with 150 mM imidazole, 50 mM sodium phosphate pH 7.2, 300 mM NaCl. Preparative gel filtration using Sephadex S200 (column volume approximately 120 mL) and eluting with 100 mM sodium

phosphate buffer at pH 7.2 was carried out, followed by concentration of the resulting protein using Amicon Ultra-4 30 kDa cutoff spin concentrators (Millipore) to an approximate concentration of 30 μ M.

The same protocol was used for apo-EryACP3 except that expression was carried out using a 500 mL LB culture in a 2L baffled flask, and elution was carried out using 500 mM imidazole, 50 mM sodium phosphate pH 7.2, 300 mM NaCl. After desalting into 100 mM sodium phosphate buffer (pH 7.2) using a PD10 cartridge (GE life sciences), the apo-EryACP3 was phosphopantetheinylated *in vitro* by incubation with sfp from *Bacillus subtilis*,^[4] (8 μ M), MgCl₂ (1 mM), DTT (5 mM) and coenzyme A (1 mM) at 37 °C for 1 h. Thrombin digestion was carried out as follows to simplify mass spectral analysis of the protein, by removing partial glucoronidation post-translational modification.^[5] Thrombin (10 μ M, from bovine plasma, Sigma) was added to the above mixture and the reaction was incubated for a further 16 h at room temperature. The holo-EryACP3 was then purified using preparative gel filtration using Sephadex S200 and eluting with 100 mM sodium phosphate buffer at pH 7.2, then concentrated using an Amicon Ultra-4 3 kDa cutoff spin concentrator (Millipore). MlsAT9 was expressed and purified by Ni-affinity chromatography following the same protocol as EryACP3. Gel filtration was carried out on Superdex75 (10/300) column (GE Healthcare) in 50 mM sodium phosphate pH 7.5, 150 mM NaCl. The purified protein was concentrated using a 10 kDa cutoff PES Vivaspin spin concentrator (Sartorius) to a concentration of 50 μ M. Protein concentration of all EryKS3AT3 variants (Σ 91890 M⁻¹cm⁻¹) and MlsAT9 (Σ 31400 M⁻¹cm⁻¹) was estimated using absorbance at 280 nm, while that of EryACP3 was estimated using amino acid analysis and Bradford's method.^[6] All EryKS3AT3 variants were freshly expressed, purified and stored for a maximum of 3 days at 4 °C prior to use, while EryACP3 was diluted to give a final glycerol concentration of 20%, flash frozen and stored for up to 2 months at -80 °C.

Chemical Synthesis

N-acetylcysteamine substrates **3-19** were prepared using the standard procedure described below, or according to literature procedures, and their identity was confirmed by comparison with literature values, except **7**, **10** and **13**, for which data are given below.^[7-14] The appropriate carboxylic acid was dissolved in anhydrous DCM, cooled on ice, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 eq) and dimethylaminopyridine (0.2 eq) were

added. After stirring on ice for 15 minutes, cysteamine (1.1 eq) was added, the reaction was allowed to warm to room temperature and stirred for 16 hours. After removal of the solvent *in vacuo*, the residue was dissolved in chloroform and washed with 0.1 M HCl followed by brine, and the organic layer was dried over MgSO₄. After removal of the solvent *in vacuo*, the residue was purified by silica chromatography using mixtures of petroleum ether and ethyl acetate.

7 96%, colourless oil, R_f 0.13 (60:40 EtOAc:petroleum ether), δ_H (CDCl₃, 400 MHz) 3.41 (2H, q, 6.3 Hz), 3.00 (2H, t, 6.3 Hz), 2.57 (1H, sex, 6.9 Hz), 1.94 (3H, s), 1.71 (1H, m), 1.46 (1H, m), 1.14 (3H, d, 6.9 Hz), 0.89 (3H, t, 7.3 Hz); δ_C (CDCl₃, 100 MHz) 204.5, 170.2, 50.1, 39.8, 28.1,

27.1, 23.1, 17.1, 11.5; *m/z* = 204.1051 [M+H]⁺, 1 ppm (204.1053 calcd for C₉H₁₈O₂N³²S);

10 73%, yellow oil, R_f 0.13 (60:40 EtOAc:petroleum ether), δ_H (CDCl₃, 400 MHz) 6.86 (1H, q, 7.1 Hz), 3.44 (2H, q, 6.0 Hz), 3.06 (2H, t, 6.4 Hz), 1.95 (3H, s), 1.87 (3H, br s), 1.83 (3H, d, 7.1 Hz); δ_C (CDCl₃, 100 MHz) 193.8, 170.2, 136.84, 136.78, 39.8, 28.3, 23.2, 14.4, 12.1; *m/z* =

202.0893 [M+H]⁺, 2 ppm (202.0896 calcd for C₉H₁₆O₂N³²S);

13 73%, white powder, R_f 0.14 (60:40 EtOAc:petroleum ether), δ_H (CDCl₃, 400 MHz) 6.89 (1H, dd, 15.4, 6.6 Hz), 6.07 (1H, dd, 15.4, 1.5 Hz), 3.45 (2H, q, 6.0 Hz), 3.08 (2H, t, 6.4 Hz), 2.46 (1H, m), 1.96 (3H, s), 1.07 (6H, d, 6.8 Hz); δ_C (CDCl₃, 100 MHz) 190.7, 170.3, 153.6, 125.7, 39.8, 31.0, 28.3, 23.2, 21.1; *m/z* = 216.1052 [M+H]⁺, 0.3 ppm (216.1053 calcd for C₁₀H₁₈O₂N³²S).

N-acylcysteamine substrate stock solutions used: **18-20** were dissolved at 60 mM in water, **6** was dissolved at 60 mM in DMSO and all other substrates were dissolved at 300 mM in DMSO.

Condensation assays using methylmalonyl-CoA

Methyl malonyl coenzyme A (1 mM), tris(2-carboxyethyl)phosphine (500 μM), EryACP3 (30 μM) and EryKS3AT3 or its mutants (3 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) for 30 mins at room temperature. *N*-acetylcysteamine substrates (6 mM) were then added and the reaction incubated at room temperature for a further hour. These reactions were carried out on a 10 μL scale, and were then quenched with 40 μL 0.1% aqueous TFA and the samples were analyzed by LCMS within 16 hours (stored at 10 °C). The reaction conditions used for **6** were adjusted to minimise precipitation observed on addition of the substrate. Methyl malonyl coenzyme A (0.4 mM), tris(2-carboxyethyl)phosphine (200

μM), EryACP3 (12 μM) and EryKS3AT3 or its mutants (1.2 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) for 1 hour at room temperature. **6** (3 mM) was added and the reaction incubated at room temperature for one hour. The reaction was carried out on a 25 μL scale, then quenched with 25 μL 0.1% aqueous TFA and analyzed by LCMS as described in the general analytical procedures.

Malonylation of EryACP3 with MIsAT9

Malonyl coenzyme A (1 mM), tris(2-carboxyethyl)phosphine (5 mM), EryACP3 (30 μM) and MIsAT9 (3 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) for 1 hour at room temperature, then quenched and analysed as above (Figure S2b). A control reaction lacking the MIsAT9 was carried out (Figure S2a). To confirm that the reaction proceeds in the presence of EryKS3AT3, the above reaction was repeated with the addition of EryKS3AT3 (3 μM), and to confirm EryKS3AT3 cannot use malonyl coenzyme A as a substrate the above reaction was carried out with EryKS3AT3 (3 μM) in place of MIsAT9 (Figures S2c and S2d).

Condensation assays using malonyl-CoA

Malonyl coenzyme A (1 mM), tris(2-carboxyethyl)phosphine (500 μM), EryACP3 (30 μM) and MIsAT9 (3 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) for 30 mins at room temperature. *N*-acetylcysteamine substrates (6 mM) and EryKS3AT3 or its mutants (3 μM) were then added and the reaction incubated at room temperature for a further hour. These reactions were carried out on a 10 μL scale, and were quenched and analyzed as for the methylmalonyl condensation assay above. The reaction conditions used for **6** were adjusted as above; malonyl coenzyme A (0.4 mM), tris(2-carboxyethyl)phosphine (200 μM), EryACP3 (12 μM) and MIsAT9 (1.2 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) for 1 hour at room temperature. **6** (3 mM) and EryKS3AT3 or its mutants (1.2 μM) were added and the reaction incubated at room temperature for one hour. The reaction was carried out on a 25 μL scale, then quenched with 25 μL 0.1% aqueous TFA and analyzed as above.

Condensation assays using alkyl-ACP as an internal standard

EryACP3 was alkylated with iodoacetamide following the protocol described by Jenner *et al.*^[15] Methyl malonyl coenzyme A (1 mM), tris(2-carboxyethyl)phosphine (500 μM),

EryACP3 (30 μM) and wild type or Ala154Trp EryKS3AT3 (3 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) for 30 mins at room temperature. *N*-acetylcysteamine substrates (6 mM) were then added and the reaction incubated at room temperature for a further hour (10 μL final volume). The reactions were quenched with 40 μL 0.1% aqueous TFA, alkylated EryACP3 was added to a final concentration of 6 μM and samples were analyzed by LCMS as described in the general analytical procedures.

Time course assays for wild type and Ala154Trp eryKS3AT3

Methyl malonyl coenzyme A (1 mM), tris(2-carboxyethyl)phosphine (500 μM), EryACP3 (30 μM) and wild type EryKS3AT3 or Ala154Trp (3 μM) were incubated in sodium phosphate buffer (100 mM, pH 7.2) at room temperature for 30 mins. Substrate **4** (6 mM) was added and aliquots (10 μL) were then extracted at various time points, quenched with 40 μL 0.1% aqueous TFA and analyzed by LCMS within 16 hours (stored at 10 °C). No significant side reactions were observed on this occasion, and since the condensation product peak was well resolved from the holo/methylmalonyl peak, estimation of peak areas was carried out spectrophotometrically (210 nm) rather than by deconvolution of LCMS data. Data was then fitted to equation 1 using OriginPro 8.^[16] Wild type EryKS3AT3 had a v_0 value of 10.6 ± 0.4 mMs⁻¹ and an η value of 0.009 ± 0.001 s⁻¹, while Ala154Trp had a v_0 value of 47.2 ± 0.8 mMs⁻¹ and an η value of 0.048 ± 0.001 s⁻¹.

$$[P] = \frac{v_0}{\eta} (1 - e^{-\eta t}) \quad (1)$$

Data analysis

The EryKS3AT3 and its mutants had an approximate retention time of 16 mins, all EryACP3 derivatives had retention times between approximately 17 and 23 mins, and MlsAT9 had a retention time of 24 mins. To allow quantification of the various EryACP3 derivatives formed during the reaction, deconvolution was carried out on the mass spectrum generated by summing all data after the EryKS3AT3, or its mutants, had been eluted and before elution of MlsAT9. The proportions of each EryACP3 derivative present were then analyzed (with a possible output range of 9000-13000 Da). In those cases where very low levels of condensation product were obtained, deconvolution of the peak containing the

condensation product was carried out to confirm its presence, and a yield of <1% was quoted. For EryKS3AT3 and all mutants, with the exception of A₁₅₄W, the most abundant ACP derivatives observed were the holo-ACP (M_w 10477) and methylmalonyl-loaded ACP (M_w 10577). Other commonly observed minor derivatives were propionoyl loaded ACP, presumed to be formed by spontaneous decarboxylation of methylmalonyl loaded ACP (M_w 10533), and ethyl loaded ACP formed by decarboxylation of malonyl loaded ACP (M_w 10519), oxidised holo-ACP (M_w 10493) and methylmalonyl-loaded ACP (M_w 10593), presumed to be oxidized on one of the two methionine side chain sulfurs, and holo-ACP that has formed a disulfide bridge with an additional molecule of coenzyme A (M_w 11240). Acylation of the unloaded holo-EryACP3 via a (non-enzyme catalyzed) trans-thioesterification reaction with the *N*-acetylcysteamine substrates was commonly observed in small quantities, while for α,β -unsaturated *N*-acetylcysteamine substrates, ACP derivatives derived from conjugate addition of the phosphopantetheine sulfur to the substrate was often a major product of the reaction (up to 77%). Neither of these reactions was observed for the apo-EryACP3. For one batch of EryACP3, a holo-ACP + 32 Da derivative was observed (M_w 10507), presumed to represent a doubly oxidised phosphopantetheine sulphur (sulfinic acid), due to the absence of a methylmalonyl loaded equivalent. While the reducing agent TCEP was included to reduce the formation of holo-ACP dimers, its concentration was minimized to avoid its conjugate addition onto Claisen condensation products when α,β -unsaturated SNAc substrates were employed.

Table S2. ESI-MS analysis of proteins

Primers for cloning EryKS3AT3 and EryACP3

EryKS3AT3 Fwd	ATATATATCATATGACTGACAGCGAGAAGGTGG
EryKS3AT3 Rev	TATATATA GAA TTGCCCTACGCGGTAGGCCAGCTCGTC
EryACP3 Fwd	ATATATATCATATGCGGCTCGGGGGCTTCC
EryACP3 Rev	TATATATA GAA TTCTAGGCCTCACCGACGAGCCGGC

Primers used for cloning of MlsAT9

MlsAT9 Fwd	AGCAGCGGCAGAACCTGTATTTCAGGGCCCCGACACCACAAACC
MlsAT9 Reverse	GCCA ACTCAGTT CCTTCGGCTTGTTAGAAAGCGTAGGTGGCAGTG
pET28a(TEV) Fwd	TAACAAAGCCC GAA AGGAAGC
pET28a(TEV) Rev	GCCCTGAAAATACAGGTTTCG

Primers used for quickchange mutagenesis of EryKS3AT3

A ₁₅₄ G sense	CTTCCTCGGAGTGGGGAAAGTTCGGCTACG
A ₁₅₄ G antisense	CGTAGCCGA ACTT CCCCACTCCGAGGAAG
A ₁₅₄ W sense	GTCTTCCTCGGAGTGT GG AAAGTTCGGCTACGG
A ₁₅₄ W antisense	CCGTAGCCGA ACTT CCACACTCCGAGGAAGAC
K ₁₅₅ A sense	CCTCGGAGTGGCG GG CTTCGGCTACGGC
K ₁₅₅ A antisense	GCCGTAGCCGA ACG CCGCCACTCCGAGG
F ₁₅₆ Q sense	CTCGGAGTGGCGAAG CA AGGCTACGGCGAGGAC
F ₁₅₆ Q antisense	GTCCTCGCCGTAGCC TTG CTTCGCCACTCCGAG
V ₁₇₃ M sense	CGAGGGCTACTCG AT GACCGGTGTGGCGC
V ₁₇₃ M antisense	GCGCCACACCGGT CAT CGAGTAGCCCTCG
A ₂₃₀ T sense	TCGGCGGTGCC AC GGTGATGGCG
A ₂₃₀ T antisense	CGCCATCACCGTGGCACCGCCGA
F ₂₆₃ T sense	CGGCGCCGACGGG AC GGCTTCTCGAA
F ₂₆₃ T antisense	TTCGGAGAAGCCGG T CCGTCGGCGCCG
F ₂₆₅ W sense	GCCGACGGGTTGGCT GG TCCGAAGGCGT
F ₂₆₅ W antisense	ACGCCTTCGG ACC AGCCGAACCGTCGGC

Table S2. ESI-MS analysis of proteins

	Calculated		Observed	
	+ Met	- Met	+ Met	- Met
EryKS3AT3	99989	99857	99992	99870
holo-EryACP3	10477	-	10477	-
A ₁₅₄ G	99975	99843	99996	99878
A ₁₅₄ W	100104	99972	100094	99973
K ₁₅₅ A	99932	99800	99940	99802
F ₁₅₆ Q	99970	99838	99957	99837
A ₂₃₀ T	100019	99887	100011	99890
F ₂₆₃ T	99943	99811	99928	99803
F ₂₆₅ W	100028	99896	100050	99909
MIsAT9	-	52288	-	52279

Values for proteins with (+ Met) and without (-Met) an N-terminal methionine are given

Figure S1. KS domain sequence alignment Sequence alignment of KS domains of known structure with a representative mycolactone KS domain. The positions of variable surface loops identified in X-ray crystal structures^[17–21] are highlighted with the ‘outer clasping loop’ in yellow, the ‘dimer interface loop’ in blue and the ‘active site cap’ in red. Regions previously identified as involved in ACP docking events^[22] are outlined in yellow for the “upstream” ACP and green for the intramodular ACP. Residues identified as possible specificity determinants^[17] are highlighted with a closed circle. Residues that when mutated obstruct the intramodular ACP docking region^[22] are indicated with open triangles. The seven EryKS3 residues mutated in this work are indicated with asterisks.

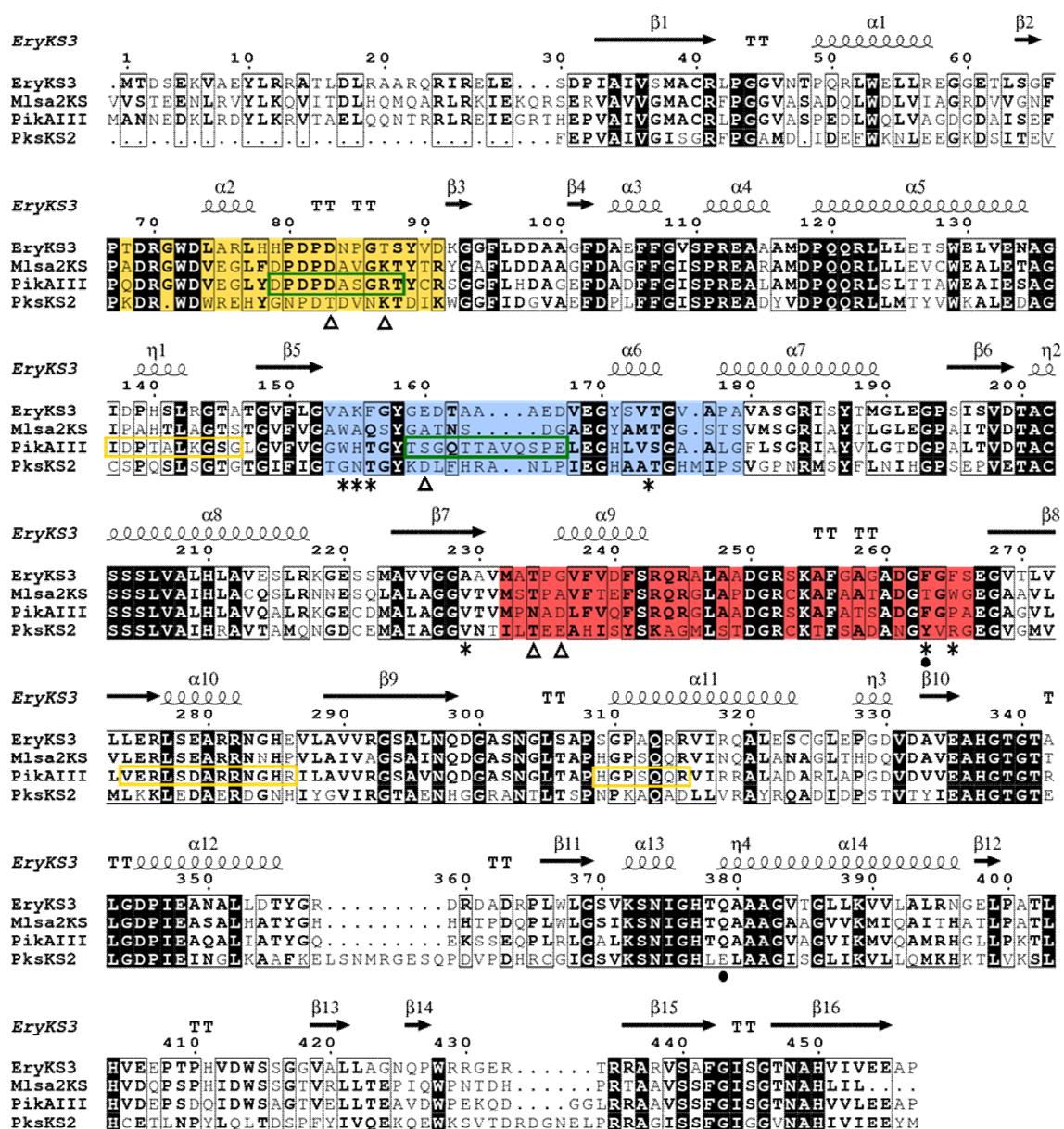


Figure S2. EryKS3AT3 crystal structure and Phyre model comparison Active site residues chosen for mutagenesis in the ketosynthase (KS) domain of Ery PKS (DEBS) module 3 (PDB accession number 2QO3) based on comparison with a Phyre2^[2] model of a representative mycolactone PKS KS (MlsA2). Conserved active site residues are shown in yellow (EryKS) or orange (MlsA2) while those that differ are shown in blue (EryKS) or green (MlsA2). Residue Val173' lies at the KS-AT dimer interface and is contributed by the other subunit. The catalytic triad is shown in red and electron density observed for the ketosynthase inhibitor cerulenin bound to the catalytic Cys is shown in purple.

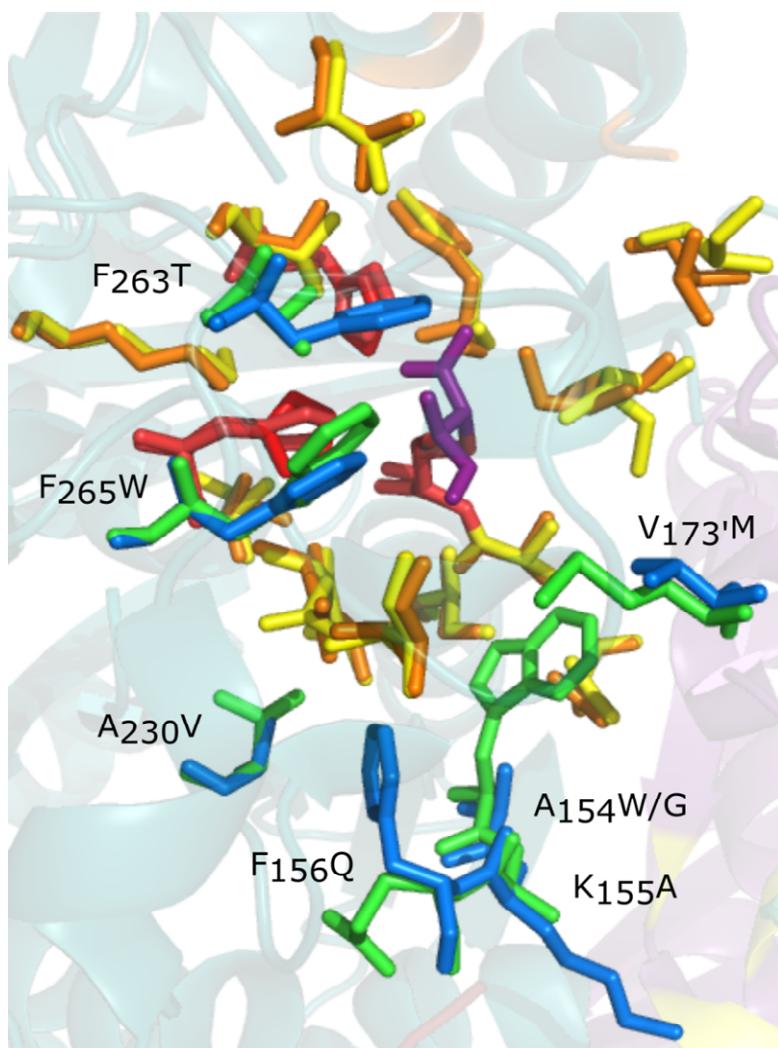


Figure S3. Sequence variability in *cis*-AT PKS domains. The percentage of sequences from 199 aligned PKS domains that have the consensus residue at each position is plotted against residue number. The regions shown in colour correspond to "variable surface loops" identified in X-ray crystal structures of both *trans*-AT and *cis*-AT KS domains.^{25,28-31} Yellow = outer clasping loop; blue = dimer interface loop; and red = active site cap.

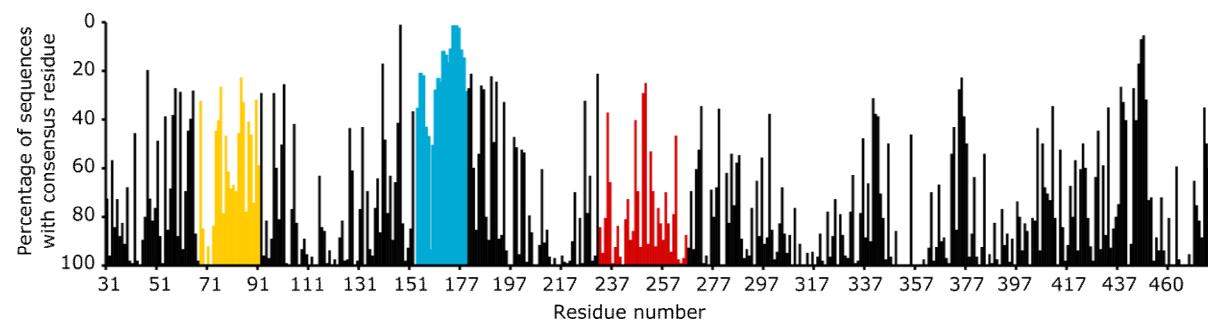


Figure S4. SDS-PAGE analysis of proteins (4-12% Bis-Tris)

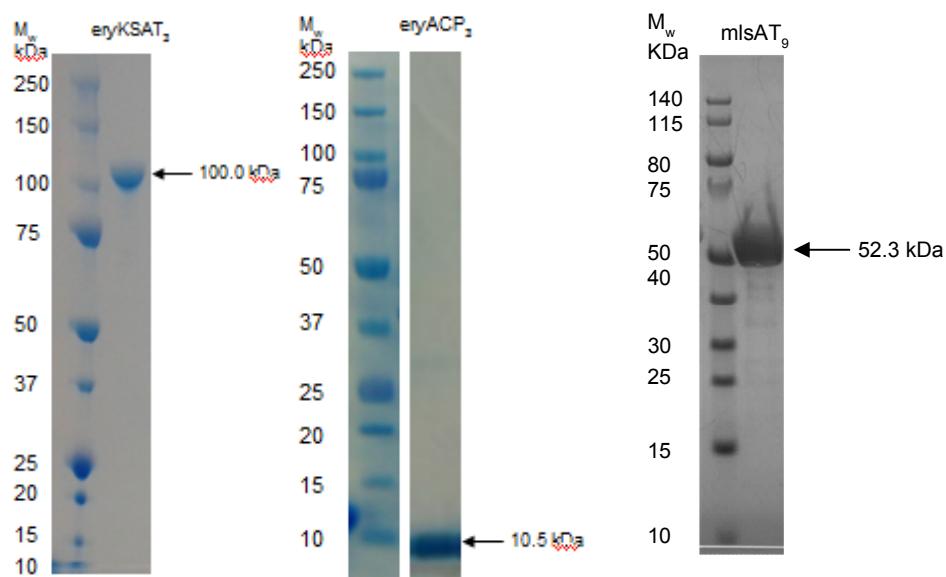


Table S3 Summary of EryACP3 condensation products

<u>Substrate</u>	<u>R_t (mins)</u>	<u>Methyl malonyl (Da)</u>	<u>Malonyl (Da)</u>
3	18.5	10604	10590
4	19.0	10618	10604
5	21.0	10660	10646
6	22.8	10688	10674
7	18.8	10618	10604
8	19.4	10644	10630
9	18.0	10602	10588
10	18.4	10618	10604
11	18.6	10616	10602
12	19.1	10630	10616
13	19.1	10630	10616
14	18.7	10628	10614
18-20	17.8	10646	10632

Figure S5. Selected MS analyses of KS-catalysed production of β -ketoacyl-EryACP3. EryACP3 after incubation with methylmalonyl-CoA (MeMal-CoA), substrate **6** and (A) wild type EryKS3AT3 or (B) Ala154Trp; ACP3 after incubation with MeMal-CoA, substrate **11** and (C) wild type EryKS3AT3 or (D) Ala154Trp; ACP3 after incubation with MeMal-CoA, substrate **13** and (E) wild type EryKS3AT3 or (F) Ala154Trp; ACP3 after incubation with MeMal-CoA, substrate **14** and (G) EryKS3AT3 or (H) Ala154Trp. Claisen condensation products for the respective substrates are shown in green, while side products (non-enzyme catalysed) arising from thioester exchange of ACP with SNAC substrate and conjugate addition of ACP onto α,β -unsaturated thioester substrates are shown in purple and orange respectively. An alkylated ACP used as an internal standard is shown in yellow.

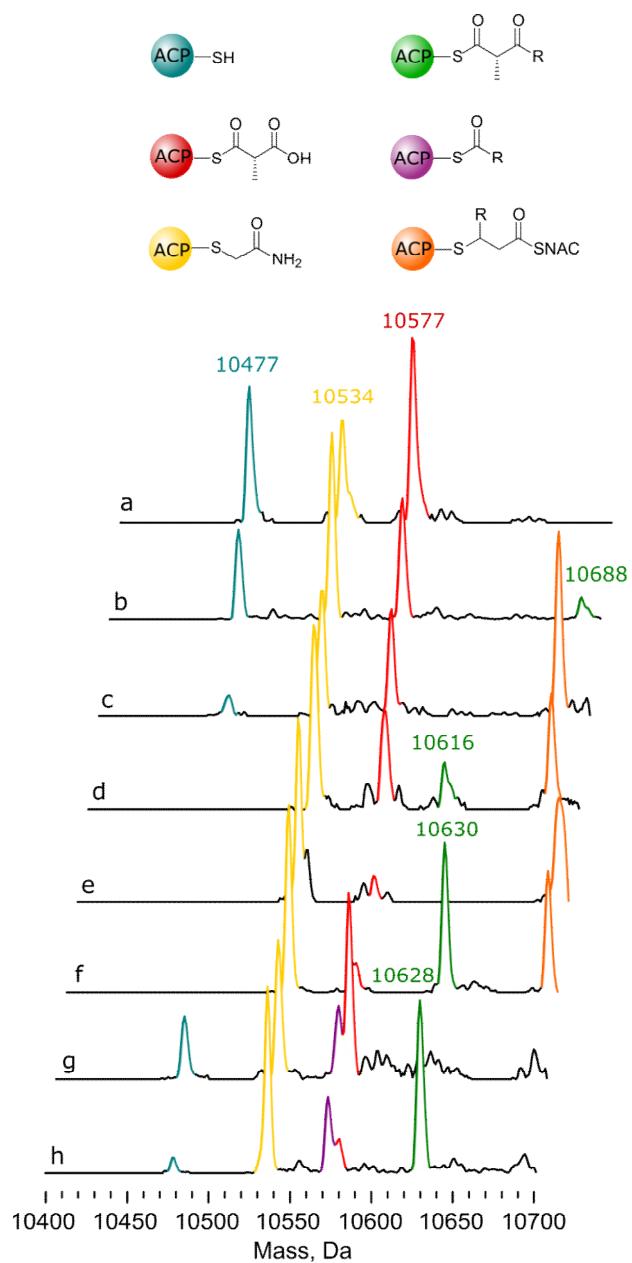


Figure S6. Time course of condensation of **4** with methylmalonyl-ACP, catalysed by wild type EryKS3AT3 and mutant Ala154Trp.

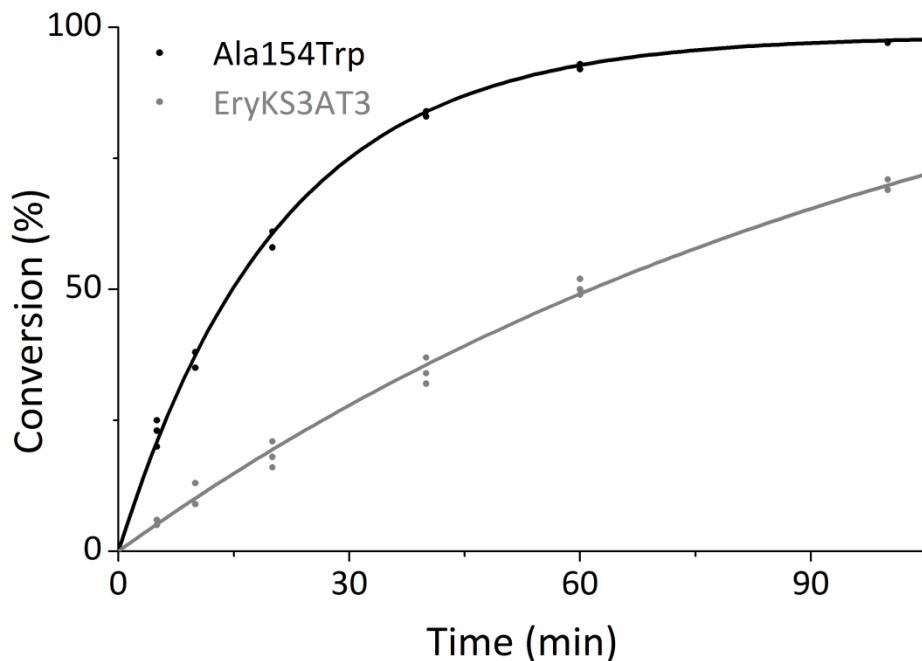


Figure S7. Malonylation of EryACP3 with MlsAT9.

Deconvoluted traces of mass spectrometric analysis of the incubation of malonyl coenzyme with EryACP3 in (a) the absence of an acyltransferase; in the presence of (b) MlsAT9; (c) MlsAT9 and EryKS3AT3; and (d) EryKS3AT3.

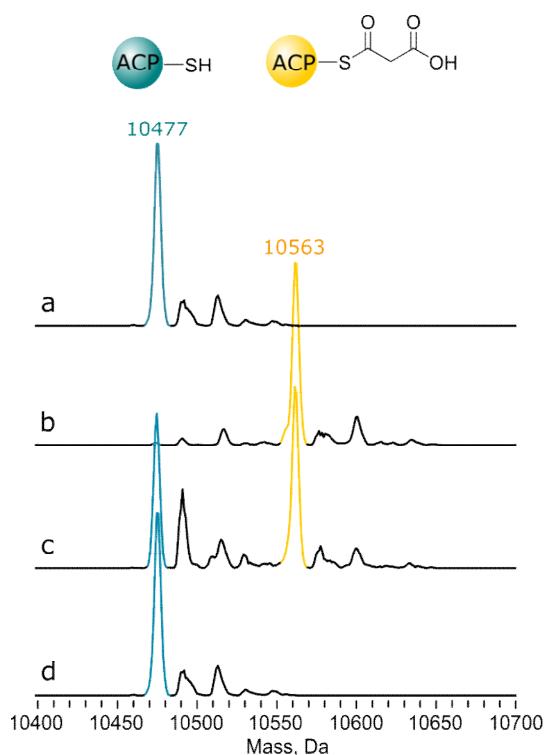


Table S4. Condensation assays using malonyl-CoA

Percentage of EryACP3 bearing Claisen condensation product after incubation with EryKS3AT3 or its mutants, malonyl CoA and synthetic SNAc substrate.

Substrate	EryKS3AT3	A ₁₅₄ G	A ₁₅₄ W	K ₁₅₅ A	F ₁₅₆ Q	V ₁₇₃ M	A ₂₃₀ T	F ₂₆₃ T	F ₂₆₅ W
3	3	T	6	5	3	3	2	T	
4	5	2	11	8	5		2	T	
5	T			6	2	2	T	T	
6				T					
7				4	2	T	T	T	
8	3			9	3	T	5	T	
9				T					
10	11	2	16	9	3	6	4		
11				3					
12				T					
13				5					
14				5	T	3			
15	T			T	4	T	T	T	
16				T					
17				T	T	T			
18									
19									

Figure S8. Dimer interface loop location

The location of the EryKS3AT3 dimer interface loop (blue sticks) including the A154 residue mutated during this study (orange) for one KS subunit is shown alongside the catalytic triad (red sticks). The surface of this subunit is shown in blue for the KS and red for the AT and post-AT linker, while the surface of the second subunit is shown in yellow.

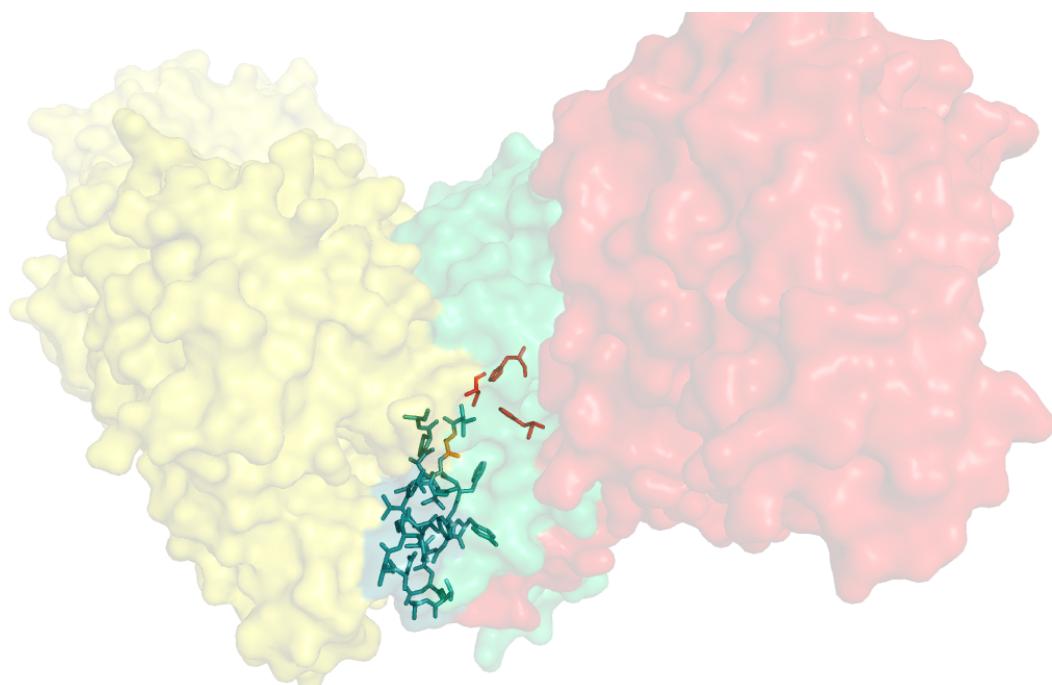


Figure S9. Sequence alignment of PKS type I *cis*-AT ketosynthases

199 sequences were chosen to represent the various type I KS clades identified by Jenke-Kodama et al., and were aligned using Clustal Omega (EMBL-EBI).^[23] All non-loading domain ketosynthases from the following clusters were used: mycolactone, erythromycin, amphotericin, avermectin, borrelidin, epothilone, lasalocid, rapamycin, nyastatin, spinosyn, tylactone, niddamycin, monensin, oligomycin, rifamycin, pikromycin and stambomycin.

mycol_001_KS_001.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_002.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_003.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_004.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_005.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_006.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_007.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_001_KS_008.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_002_KS_001.seq	ERVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_001.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_002.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_003.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_004.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_005.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_006.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
mycol_003_KS_007.seq	EPVAVVGMACRFPGGVASADQLWDLVIAGRDPVGNFPADRGWDVEGLFDPD-PDAVGKTY
eryth_001_KS_001.seq	EPVAVVAMACRLPGGVSTPEEFWELLSEGRDAVAGLPTDRGWLDLSFHPD-PTRSGTAH
eryth_001_KS_002.seq	EPIAIIVGMACRLPGEVDSPERLWELITSGRDSAEEVPDDRGWVPDELMASD-AA---GT
eryth_002_KS_001.seq	DPIAIIVSMACRLPGGVNTPQLWLREGGETLSGFPDRGWDLARLHHPD-PDNPGTSY
eryth_002_KS_002.seq	EPIAIIVGIGCRFPGGIGSPEQLWRVLAEGANLTTFGFPADRGWDIGRLYHPD-PDNPGTSY
eryth_003_KS_001.seq	EPIAIIVGMACRFPFGDVSPESFWFVSGGDDAIAEAPADRGWEPEP-----D
eryth_003_KS_002.seq	DPIAIIVGMACRFPGGVHNPGELWEFIVGRDAVTEMPTDRGWLDALFDPD-PQRHGTSY
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ampho_002_KS_002.seq	--IVIVGMSCRFPGGANTPPEELLRLALDGADVISEFPA DRGW DAHGLYHPD-PDRQGRTY
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ampho_003_KS_004.seq	DPIVIVGMACRFPGGITSPEDLWRLLIGEFGSDVISDFPVNRGWDVD SLYDPD-PAHTGTSY
ampho_003_KS_005.seq	DPIVIVGMACRFPGGVASPEDLWRLVTEGTDAVSAFPVN RGWDVENLYHPD-PDNIGTSY
ampho_003_KS_006.seq	DPIVIVGMACRFPGGVASPEDLWRLLLEGDAITEF PVNRGWDIESVYHPD-PEHLGTSY
ampho_004_KS_001.seq	EPIAIIVGMC RYPGVSSPEDLWRMVEAGEENGVTPTDRGW DLEA LASS-----P
ampho_004_KS_002.seq	DPIVIVGMACRYPGGVTSPEDLWRLVTEGTDAVSGFPVN RGWDV ENLYHPD-PDH PG TAY
ampho_004_KS_003.seq	EPIAIIGMSCRYPGVSSPEQLWDLVLSGTD AITDFPVNRGWN TAGLYDPD-PDH PG TTY
ampho_004_KS_004.seq	DPIAIIVGMSCRYPGGITSAEQLWRVSLEEVDAVSVFPA DRGW DAEALYDPD-PDASGRTY
ampho_004_KS_005.seq	DPIAVVGLACRFPGGVSTPEELWQLIAEGRD GITGFPPDRRNW DIAALGAGA-----SD
ampho_004_KS_006.seq	DPIVIVGMACRFPGGVNSPEDLWQLVLDEVAGDFPDRGWLD ALAGDG-PG-RSAT
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ampho_005_KS_002.seq	DPVIVVGMGCRFPGGVGSPEDLWDLIASGSDAITGFPA DRWE S ST-IGGE--P-GDLS
ampho_005_KS_003.seq	DPVIVVGMGCRFPGGVGSPEELWDLV ASGTDAITGFPA DRWE S ST-IGGE--P-GDLS
ampho_006_KS_001.seq	EPVAIVGMACRFPGGVRSPEDLWEMIADGRDAISGFPA DRGW DLELAGDG-AG-GSST
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averm_002_KS_001.seq	EPIAIIVGMACRYPGGATSPTRLWHLVKQSQTDAIGGFPTDRGW NLEQLYDPD-PDRSGTSY
averm_002_KS_002.seq	EPIAIIVGMACRYPGVRTVDLWLVSGGHDAIGGFPTNRG WDLD TLYNPD-PDH HGTSY
averm_002_KS_003.seq	EPIAIIVGMACRFPGGVTSADD FWDLISSE QDAIGGFPTDRGW DLD TLYDPD-PDH PG TCY
averm_002_KS_004.seq	EPIAIIGMACRFPGGVRSADD LWELLASGKDAIGVFP TDRGW DLD TLYDPD-PDH PG TCY
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 ADEGAFLPDAGDFDAAFFGINPREALAMDPQQRLLLEASWEVLERAGIDPTTLKG-TG
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 VRDGAFLYDAGHFDAAFFGISPRAEVAMDPQQRLLLETWEAIEHAGMNPHALKSD-TG
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 rifam_005_KS_002.seq TSRGGFLDGAGLFDAGFFGISPREALAMPQQRLLLEAAWEALEGTGVDPGLKGAD-VG
 pikro_001_KS_002.seq VRQGGFIENVAGFDAAFFGISPREALAMPQQRLLLETSWEAVEADAGIDPTSLRGRQ-VG
 pikro_001_KS_003.seq VREGGFLHDAAEFDAEFFGVSPREALAMPQQRMLLTTSWEAFAERAGIEPASLRGSS-TG
 pikro_002_KS_001.seq AREAGFLDEAGEFDADFFGISPREALAMPQQRLLLETSWEAVEAHAGIAPATARTGS-TG
 pikro_002_KS_002.seq CRAGGFLEAGEFDADFFGISPREALAMPQQRLLLETSWEAVEADAGIDPTSLQGQQ-VG
 pikro_003_KS_001.seq CRSGGFLHDAGEFDADFFGISPREALAMPQQRLLSLTTAWEAIESAGIDPTALKGSG-LG
 pikro_004_KS_001.seq VRNAAFLDDAAAGFDAAFFGISPREALAMPQQRQLLEASWEVFERAGIDPASVRGTD-VG
 Stamb_001_KS_002.seq PTAGGFLLDDIAFGFDAALFGISPRAEVAMDPQQRLLLEVTEAWEALERLGTDPDLRGRS-TG
 Stamb_001_KS_003.seq ARHGGFLHDAADFDAELFGISPRAEALAMPQQRLLLEISWEAFAERAGIDPTGLRGSD-TG
 Stamb_001_KS_004.seq ARGGFLPDAAGFDADLFGVSPREAQAMPQQRMLIESETFERAGVDPASLQGSR-VG
 Stamb_002_KS_001.seq GHEGGFLHEAAEFDAGLFEISPRAEVAMDPQQRLLLETSWEVFERAGIDPKSVRGAR-VG
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 Stamb_003_KS_002.seq ARGGFVGDGATEFDAALFGISPRAEALAMPQQRLLLEASWEAFAEHAGIDPLSQNHTR-TG
 Stamb_003_KS_003.seq AARGGFLEDEATFDAALFGISPRAEALAMPQQRLLLETAWEAFESAGIATSDAHSS-TG
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 Stamb_004_KS_001.seq ARQGGFVHDATAFDAFFGVSPREALAMPQQRLLLEAWEVFEHAGIDPQSVRGTR-TG
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 Stamb_005_KS_001.seq APRGGFVHDVGFDFDAGLFGISPRAEATDPQQRQLLEASWEAVERAGIDMRTLRGGR-TG
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 Stamb_007_KS_001.seq VREGGF柳HDAGEFDAGLFGISPRAEATDPQQRMLLETSWEAFAERAGIDPLSLRGRS-TG
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 Stamb_009_KS_002.seq PRTGGFLPDATFDAELFGISPRAEALAMPQQRLLLEASWEAFAERAGIHPRSLAGSR-TA
 Stamb_009_KS_003.seq TRSGGF柳DDIAAFDAGLFGISPRAEALAMPQQRMLLEASWEAFAERAGIDPTSVRRSD-TG
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 VFVGNSQDYGTLRLR---ADDRSHAYLATGASASVLSGRISYTLGLEGPAPTIISTAC
 VYAGVMYHEYASRLGA---TPAGFEGTJGTGSSGSIASGRISYTFDLTGPAPTVDTAC
 VYTGLMTHEYATRLPS---IDEELEGVIGIGNAGSVASGRVSYTLGLNGPAVTVDTAC
 VYVGAWNSNYGRGG----GAESSEGHLLTNASSVSVSGRVAVGLEGPAVTIDTAC
 VWFGTIGQDYFSLFAASG---GEHANYLACASASVMSGRVSYVLEGPAVTVDTAC
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 VFAGAWE SGYQ KGL DAAD-----AGLE AQLLAGI-VSFTAGR VSYT LGLEGPA M TV DTAC
 VFVGA AHGTG YASD PARAP-----EGTEG YLLTG DADAVL SGR IAY VL GLEGPA ITV ETAC
 VFVGM TGQDY GPR-LHE-----PSQATDGYLLTG STPSV ASGR LSFS FGLEGPA LTV DTAC
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 VITGIMYDDY GS RFLAR-----KPDGF EGR IMTGST PSV ASGR VAYTF GLEGPA ITV DTAC
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 pikro_002_KS_002.seq ADARLTADVDV DVVEAHGTGTRLGDPIEAQALIATYQGQRDTEQPLRLGSILKSNI GHHTQA-
 pikro_003_KS_001.seq ADARLAPGDV DVVEAHGTGTRLGDPIEAQALIATYQGEEKSSEQPLRLGALKSNI GHHTQA-
 pikro_004_KS_001.seq ADARLTSSDV DVVEGHGTGTRLGDPIEAQALLATYQGQRAPGQPLRLGTLKSNI GHHTQA-
 Stamb_001_KS_002.seq DAAGLRPSD VDVMVEAHGTGTRLGDPIEAQALLATYQDQERDAERPLWLGSILKSNI GHHTQS-
 Stamb_001_KS_003.seq ADAGLAPGDV DMVEAHGTGTLGDPVEAEALLATYQV RDAERPLWLGSILKSNI GHHTQA-
 Stamb_001_KS_004.seq NSAGLWPSEV DVAEAHGTGTRLGDPIEAQALLATYGRDRS --EPLYLGGSVKSNI GHHTQA-
 Stamb_002_KS_001.seq ASAGLAPS DVLVEAHGTGTRLGDPIEAQALLA YGRNRAAGHPLWLGSVKSNI GHHTQA-
 Stamb_002_KS_002.seq ANARLEASDV DVVEAHGTGTRLGDPIEAQALLATYQGQRHEDRPLWLGSVKSNI GHHTQS-
 Stamb_003_KS_001.seq ANAQLTTDV DVAEAHGTGTLGDPIELEAL QDTYGRDR DDRPLWLGSILKSNI GHHTQA-
 Stamb_003_KS_002.seq ANARLEASDV DVVEAHGTGTRLGDPIEAQALLATYQGQRDEDRPLWLGSVKSNI GHHTQA-
 Stamb_003_KS_003.seq ANARLAASEV DVAEAHGTGTRLGDPIEAQALLATYQGQRDEDRPLWLGSVKSNI GHHTQA-
 Stamb_003_KS_004.seq ATAGLAPGEV DVVEAHGTGTRLGDPIEAQALLATYQGQRDEDRPLWLGSVKSNI GHHTQA-
 Stamb_003_KS_005.seq ADARLSPSEV DVAEAHGTGTRLGDPIEAQALLATYQDQR DADRPLWLGSILKSNLGHTQA-
 Stamb_004_KS_001.seq ADARLSASDV DVVEAHGTGTRLGDPIEAQALLA YQDQRPEGRPLLGSVKSNI GHQA-
 Stamb_004_KS_002.seq ADAGLTDADVDVAEAHGTGTLGDPIEVQALM ATYGRDRDEE QPLWLGSILKSNI GHHTQS-
 Stamb_004_KS_003.seq ANSGLDAADIDTVEAHGTGTLGDPIEAQSI LATYQGQRDEGRPLWLGSVKSNI GHHTQS-
 Stamb_005_KS_001.seq ASAALVPAEV DVAEAHGTGTLGDPIEAQALLATYQDQR --EPLYLGSI KSNI GHHTQA-
 Stamb_006_KS_001.seq AAARLSAAEIDVAEAHGTGTLGDPIEAHALLATYQDQR --QPLYLGSILKSNI GHHTQA-
 Stamb_006_KS_002.seq ADADLRHS DDV DVVEAHGTGTLGDPIEAQALLATYQDQR --EPLYLGSI KSNI GHHTQA-
 Stamb_007_KS_001.seq ENARLAAADIDV DVVEAHGTGTLGDPIEAQALLNAYQDQR PADRPLWLGSILKSNI GHQA-
 Stamb_007_KS_002.seq ATAGLAPHEV DVLVEAHGTGTLGDPIEAQALLD VYQDQR PERPLWLGSILKSNI GHQA-
 Stamb_008_KS_001.seq DDARLTPDQDV DVVEAHGTGTLGDPIEAQALLATYQGRDR --DRPLWLGSILKSNI GHQS-
 Stamb_008_KS_002.seq AAARLTAADIDV DVVEAHGTGTLGDPIEAQALLNAYQDQR PADRPLWLGSILKSNI GHHTQA-
 Stamb_009_KS_001.seq AAARLTAADIDV DVVEAHGTGTLGDPIEAQALLNAYQDQR PADRPLWLGSILKSNI GHHTQA-
 Stamb_009_KS_002.seq ASAGLEATDV DVLVEAHGTGTRLGDPIEAEALLATYGRDRS --EPLYLGGSVKSNI GHHTQA-
 Stamb_009_KS_003.seq ADARL TAAEV DVAEAHGTGTLGDPIEAQALLATYQGQR --EPLYLGGSVKSNI GHHTQA-
 Stamb_009_KS_004.seq AAAGLEAAEV DVAEAHGTGTLGDPIEAQALLATYQDQR --EEPLYLGGSVKSNI GHHTQA-
 : * * * : * * * : * . . : * : * .. *

mycol_001_KS_001.seq AAGAAGVVVKM IQAITHATLPAT-LHV DQPSPHIDWSSGT VRL LTEPIQW-PNTD---HPR
 mycol_001_KS_002.seq AAGAAGVVVKM IQAITHATLPAT-LHV DQPSPHIDWSSGT VRL LTEPIQW-PNTD---HPR
 mycol_001_KS_003.seq AAGAAGVVVKM IQAITHATLPAT-LHV DQPSPHIDWSSGT VRL LTEPIQW-PNTD---HPR
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 mycol_001_KS_008.seq AAGAAGVVVKM IQAITHATLPAT-LHV DQPSPHIDWSSGT VRL LTEPIQW-PNTD---HPR
 mycol_002_KS_001.seq AAGAAGVVVKM IQAITHATLPAT-LHV DQPSPHIDWSSGT VRL LTEPIQW-PNTD---HPR

mycol_003_KS_001.seq AAGAAGVVVKM1QAIATHATLPAT-LHVDPSPSPHDWSSGTVRLLTEPIQW-PNTD---HPR
mycol_003_KS_002.seq AAGAAGVVVKM1QAIATHATLPAT-LHVDPSPSPHDWSSGTVRLLTEPIQW-PNTD---HPR
mycol_003_KS_003.seq AAGAAGVVVKM1QAIATHATLPAT-LHVDPSPSPHDWSSGTVRLLTEPIQW-PNTD---HPR
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eryth_001_KS_001.seq AAGVAGVIKVLAMRAGTLPLRT-LHASERSKEIDWSSGAISLLEDEPEPW-PAG---ARPR
eryth_001_KS_002.seq AAGVAGVIKVLGLERGVVPPM-LCRGERSGLIDWSSGEIELADGVREWSAAD---GVR
eryth_002_KS_001.seq AAGVTGLLVVLAIRNGELPALT-LHVEEPTPHWDWSSGGVALLAGNQFW-RRG---ERTR
eryth_002_KS_002.seq AAGAAGVIKVVLAMRHGMLPRS-LHADELSPHIDWESGAVEVLRREEPVW-PAGE---RPR
eryth_003_KS_001.seq AAGVAGVMKAVLRLRGEMPRT-LHFDEPSHQIEWDLGAVSVVSQARSW-PAG---ERPR
eryth_003_KS_002.seq AAGVAGVIKVLGLNRLGVPPM-LCRGERSGLIPIEWSSGGVELAEPWPWAADD---GVR
ampho_002_KS_001.seq AAGVAGLVKVMAMAHNGTLPLRT-LHLTEPSTHWDWSLGAVRLLTEETAW-PETG---RVR
ampho_002_KS_002.seq AAGVAGVIKVMAMHAGELPGT-LHI DEPSSHVDWTSGAVTLLRERTEW-PAVG-D-RPR
ampho_003_KS_001.seq ASGAAGVIKMIMAIQHGVLPRS-LHAERPTSNVDWTAGAVELLDRADW-PET---GRAR
ampho_003_KS_002.seq AAGVAGVIKMTMSMRHGLLPQT-LHVDAPSSHVDWDAGAVELLTEQTEW-PEN---DRV
ampho_003_KS_003.seq AAGVAGVIKVMMSMRHGVLPC-LHVDAPSSHVDWTGEGAVELLTEQTEW-PET---DRV
ampho_003_KS_004.seq AAGAAGVIKVMMSMRHGVLPRS-LNISEPSSHVDWSAGAVELLTEQTEW-PET---DRV
ampho_003_KS_005.seq AAGVAGVIKVMMSMRHGVLPR-T-LHVDAPSSHVDWTGEGAVELLTEQTEW-PET---EHVR
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ampho_004_KS_004.seq ASGVASI1KLVHALREGVAKPS-LHIDQPSHTHWDWSSGTIQLLTERTEW-PETG---RPR
ampho_004_KS_005.seq AAGVAGVIKVMALMQHGTLPLRT-LHVTSPSTSVDWSSGAVSLLTEERDW-PETG---RPR
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ampho_005_KS_003.seq AAGAAGLVKVMAMHQGTLPLRT-LHVTEPSTHWDWSLGAVRLLTEETAW-PETG---RVR
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References

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