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Mutant Malonyl-CoA Synthetases with Altered Specificity for Polyketide Synthase Extender Unit Generation

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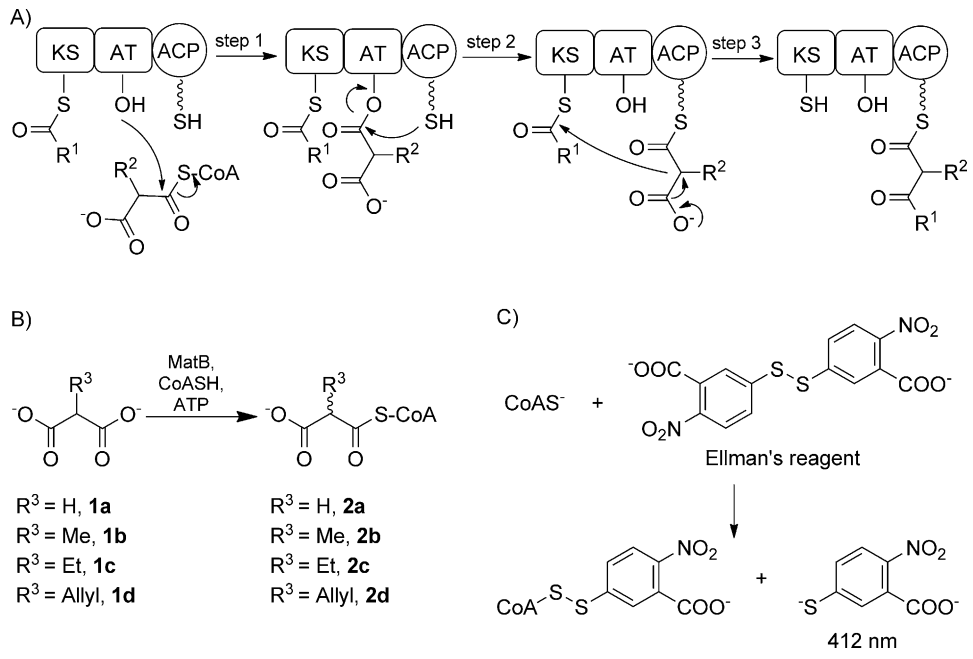
Polyketides are a large group of structurally diverse secondary metabolites with significant therapeutic utility.[1] The polyketide scaffolds of natural products are constructed by the repetitive decarboxylative condensation of small molecule building blocks called extender units, through the action of polyketide synthases (PKSs). A large portion of these PKSs are known as modular PKSs, whereby each module contains the domains required for condensation and processing of the extender unit (Scheme 1).[2–4] The pool of extender units available for PKSs in the cell, however, is limited. For example, malonyl-coenzyme A (CoA) and (2*S*)-methylmalonyl-CoA are the most common extender units. Less common extender units include methoxy-, hydroxy-, and aminomalonyl-acyl carrier proteins (ACP), which are provided by biosynthesis while bound to ACP.[5] It is this range and subsequent exquisite selection of extender units by PKS modules that is at least partly responsible for the enormous diversity of polyketide structure.

Driven by the necessity to produce milligram quantities of both natural and non-natural extender units for probing the activity of PKSs,[6–8] enzymatic approaches by using the malonyl-CoA synthetase MatB from *Rhizobium trifolii* and *Streptomyces coelicolor* have been reported.[9,10] These enzymes provide an alternative to the usual carboxylative route to malonyl-CoA, and provide substrates for fatty acid biosynthesis and the citric acid cycle.[11] However, although MatB has broad substrate tolerance, activity towards several substrates is very low or non-detectable, and the enzymes require further optimization. For example, activity with ethyl-, dimethyl-, and isopropyl-malonate are all below 10% of the activity with malonate.[10] The catalytic proficiency of MatB might limit the efficiency of artificial pathways engineered for the synthesis of polyketide analogues, particularly when non-natural extender units and/or noncognate PKS domains/modules are employed in combinatorial biosynthesis experiments.[2,12,13] In addition, engineered MatB synthetases could also prove useful for the generation of various CoA derivatives for biofuel and other synthetic biology projects.[14,15]



Scheme 1. Polyketide biosynthesis and the role of MatB. A) Extender units are selected by acyltransferase (AT) domains (step 1) and subsequently loaded onto acyl carrier protein (ACP; step 2). Ketosynthase domain (KS) then catalyzes the decarboxylative Claisen condensation between the ACP-bound extender unit and donor ketide already bound to the KS (step 3). Typically, R^2 of the extender unit is –H or –Me, although examples of incorporation of methoxy-, amino-, ethyl-, and hydroxymalonate are emerging. B) Reaction catalyzed by MatB: the natural substrate of MatB is malonate (**1a**). C) For high-throughput screening of MatB activity toward non-natural malonate analogues, activity is reported by treatment of remaining CoASH with Ellman's reagent; CoA: coenzyme A.

Encouraged by the success of directed evolution approaches aimed at altering the substrate specificity of enzymes,[16–19] we have initiated an enzyme engineering program aimed at producing variants of the *R. trifolii* MatB with altered substrate specificity. The recently solved crystal structure of the *S. coelicolor* homologue indicated that two active site residues, Val188 and Met291, could be important for controlling specificity toward the malonate α-position side-chain.[9] Amino acid sequence alignment revealed that a Thr and Met were present at the equivalent positions in the *R. trifolii* enzyme (see the Supporting Information). Accordingly, these two residues (Thr207 and Met306) were selected for saturation mutagenesis to afford the T207X and M306X libraries.

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Critical to the success of directed evolution based approaches for enzyme engineering is the availability of a suitable high-throughput screen. We reasoned that Ellman's reagent could be used to monitor the concentration of CoASH remaining after MatB reaction,[20,21] in an end-point fashion. Consumption of CoASH would result in a decrease in absorbance at 412 nm after treatment with Ellman's reagent. Indeed, lysates prepared from *E. coli* cell cultures that over-expressed MatB showed a small amount of activity with **1b**, compared to lysates prepared from the negative control, which harbored pET28a (Figure 1), as indicated by the lower absorbance of the

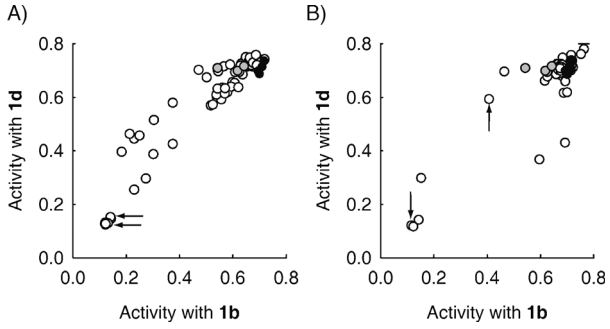

Figure 1. Activities of saturation mutagenesis libraries. Individual bacterial colonies from each library were inoculated into the wells of a 96-deep-well microtitre plate. After being cultured the protein was expressed and cells were lyzed, in situ. Samples of lysate were then assayed for the consumption of CoASH with **1b** or **1d** in the presence of ATP. Activity was measured as the final absorbance at 412 nm and was not corrected for protein expression level or cell density. The activity of several WT MatB (●) and negative control (pET28a, ●) colonies are shown for comparison. A) Colonies from the T207X library. B) Colonies from the M306X library. Arrows indicate positions of representative T207S and M306I clones in each library.

MatB samples versus pET28a. The methyl- and allylmalonate analogues (**1b**/**1d**) were initially chosen given their close structural similarity to the natural substrate, **1a**. We hoped that once mutants improved toward these diacids were identified, activity toward other more sterically demanding analogues (e.g., benzyl-, isopropylmalonate) could be established. In contrast, the activity of wild-type (WT) MatB towards **1d** in crude extracts could not be detected under our assay conditions, as compared to pET28a lysates. These preliminary studies confirmed **1b** as a substrate for MatB, and validate the colorimetric-based assay.

Subsequently, approximately 300 colonies from each of the T207X and M306X libraries were screened with **1b** or **1d** by using the colorimetric-based MatB assay. The mean absorbance of WT MatB lysates with **1b** as substrate was 0.61 with a standard deviation (SD) of approximately 7%; thus, any library member with activity lower than 0.48 (3×SD) was considered more active than WT MatB. By these criteria, colonies from both libraries showed improvements in activity toward both substrates, as indicated by final absorbance values that were lower than that for the WT MatB control (Figure 1). Thr207 seemed more accepting to mutation than Met306, in terms of activity with each substrate. Interestingly, library members were significantly improved towards *both* substrates, indicating that substitutions at positions 207/306 could broaden specificity, rather than shift specificity to a single substrate.

Several colonies that showed improved activity in these primary screens were selected for further analysis. These library members were grown on a larger scale and extracts were prepared for the colorimetric MatB assay. Subsequent confirmed hits were submitted for DNA sequencing. This analysis revealed the mutations T207S and M306I were responsible for improved hits from the T207X and M306X library (data not shown).

Each of these two mutants, along with the WT enzyme, was then purified to homogeneity for determination of specific activity with **1b**/**1d** by using a more rigorous HPLC-based enzyme assay. Briefly, this assay involves quenching MatB reaction mixtures at timely intervals, and analyzing the product mixture by RP-HPLC. The resulting CoA thioester is easily detected at 254 nm and is well-separated from the remaining CoA (Figure 2). The peak-area of each putative CoA thioester was converted to concentration by using a calibration curve established with a commercial standard of **2b** (see the Supporting Information). We fully expect this calibration curve can be used for a broad range of acyl-CoA products (see the Supporting Information), given that the detection wavelength reports the CoA portion of the acyl thioester.[10] The diacids (**1b**–**1d**) were not detected under these conditions. In addition to testing **1b**/**1d**, we reasoned that the intermediate size of the ethyl substituent of **1c** (Scheme 1) compared to methyl- and allylmalonate might be accommodated by these MatB mutants, and included this diacid in our analysis, even though it was not an original target of our screening.

Product identity in each case was confirmed by LC/MS analysis (Figure 2), and by comparison to product standard in the case of **2b** (see the Supporting Information). The T207S and M306I mutants out-performed the WT enzyme with malonate derivatives **1c** and **1d** (Figure 2). Notably, the specific activity of T207S and M306I was 70- and 50-fold improved toward ethylmalonate **1c**, compared to WT MatB. Both mutants also showed modest improvements in activity with **1d**, while under these assay conditions, activity toward **1b** was largely unchanged compared to the WT enzyme. These data suggest that the specificity of T207S and M306I was very different from the WT enzyme. Accordingly, both mutants were chosen for a detailed kinetic analysis with each substrate, along with the WT MatB, by using the HPLC-based assay. Kinetic parameters of the WT enzyme were in good agreement with literature values.[10,22] The WT enzyme displayed a strong preference for methylmalonate **1b**, with an approximately 40- and 16-fold lower specificity constant (k_{cat}/K_M) toward **1c** and **1d**, respectively (Table 1 and the Supporting Information). However, in stark contrast, the specificity constant of mutant T207S was improved 1.7-, 81-, and 31-fold, toward **1b**, **1c**, and **1d**, respectively, as compared to WT MatB (Table 1). Remarkably, the single mutations, T207S and M306I, each resulted in a moderate preference for the ethyl analogue **1c**, with a specificity shift of approximately 50- and 240-fold toward **1c** from **1b**, respectively. Cumulatively, these data confirm the critical role that Thr207 and Met306 play in controlling the substrate specificity of MatB.

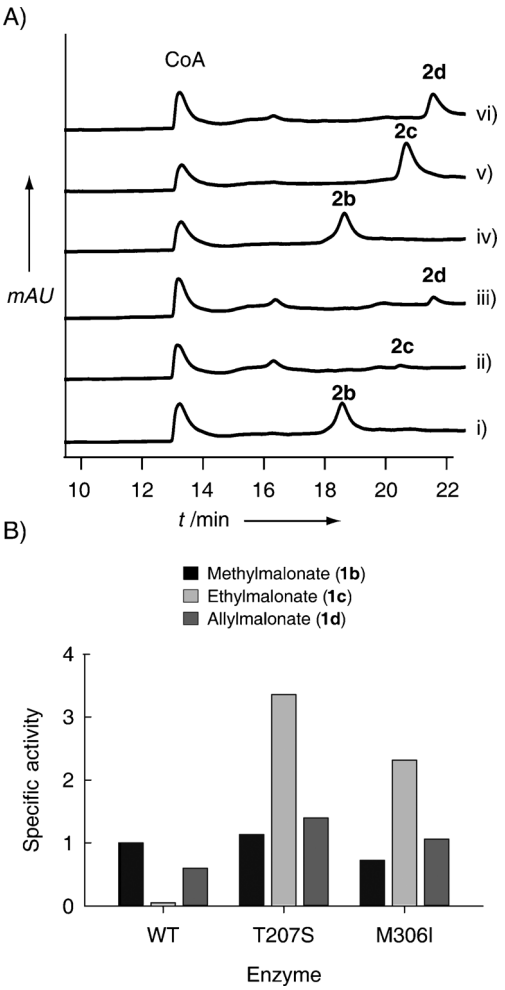

Figure 2. Specific activities of WT and mutant MatB. A) RP-HPLC analysis of MatB reactions: i) WT MatB, **1 b**; ii) WT MatB, **1 c**; iii) WT MatB, **1 d**; iv) M306I, **1 b**; v) M306I, **1 c**; vi) M306I, **1 d**. See the Experimental Section for details. Identities of putative product peaks were confirmed by negative mode QTOF MS with observed masses (calculated masses) for **2 b**, **2 c**, and **2 d** of 866.10 (866.12), 880.11 (880.14), 892.11 (832.14). Identity of **2 b** was also confirmed by co-elution with a commercial standard (see the Supporting Information). B) Specific activities of the WT and mutant MatB enzymes. Specific activities were determined by RP-HPLC as described in the Experimental Section. Standard error of these measurements is ±10%. The specific activity of WT MatB with **1 b** was set to 1.

The substrate range of WT MatB is reported to include dimethyl-, isopropyl-, and benzyl-malonate.[10] However, using either WT, T207S, or M306I MatB, we failed to confirm production of the corresponding CoA product, by LC/MS analysis, although very small peaks (<1% activity with **1 b**) by RP-HPLC could be observed (data not shown). We note that for previous specificity studies very high concentrations of diacid were utilized to provide relative rates.[10]

To the best of our knowledge, this study represents the first demonstration of tailoring MatB substrate specificity. Given the rapid success afforded by our focused random mutation and screening strategy, we hope to generate MatB mutants with improved activity toward other poor or nonsubstrates. For example, error-prone PCR, by using T207S as template, and subsequent screening with our colorimetric assay, could identify new mutations that tailor specificity toward a given diacid, or those that result in further broadening specificity. Mutant MatB enzymes with specificity towards completely non-natural malonate analogues will be very powerful tools for providing extender units to probe the specificity and activity of PKS modules/domains and related enzymes. Further, coupling the activity of engineered MatB mutants with other engineered enzymes, such as the stand-alone malonyl-CoA:ACP transacylase,[23] might provide unique opportunities to alter the backbone scaffolds of polyketides. We believe that simple spectrophotometric screens, such as that reported here, will prove effective to alter the specificity or activity of other enzymes relevant to polyketide biosynthesis.

Experimental Section

General: Unless otherwise stated, all materials and reagents were of the highest grade possible and purchased from Sigma. Isopropyl β-D-thiogalactoside (IPTG) was from Calbiochem (Gibbstown, NJ, USA). Bacterial strain *E. coli* BL21(DE3)pLysS competent cells was from Promega (Madison, WI, USA). Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA).

Cloning of MatB into pET28a: The gene for MatB from *R. trifolii* was synthesized by GeneScript, and was then subcloned into pET28a through the NdeI and HindIII restriction sites.

Saturation mutagenesis: T207X and M306X libraries were prepared by using the "round-the-horn" site-directed mutagenesis method,[24] with pET28a–MatB as template. Each subsequent ligation reaction was transformed directly into NovaBlue competent cells (Novagen). Then, all the transformants from each library were pooled and cultured, overnight. Plasmid was prepared from this

Table 1. Steady-state kinetic parameters of WT and mutant MatB enzymes.[a]

	Substrate[b]								
	Methylmalonate (**1 b**)			Ethylmalonate (**1 c**)			Allylmalonate (**1 d**)		
	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [s^{-1} mM^{-1}]	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [s^{-1} mM^{-1}]	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [s^{-1} mM^{-1}]
wild-type	2.44±0.38	0.12±0.06	21	0.61±0.08	1.12±0.3	0.54	1.86±0.15	1.49±0.25	1.30
T207S	2.13±0.2	0.06±0.02	35.5	6.11±0.62	0.14±0.04	43.6	5.30±0.82	0.13±0.07	40.80
M306I	2.05±0.3	0.44±0.13	4.7	4.91±0.52	0.17±0.05	29	3.42±0.69	0.55±0.28	6.20

[a] Steady-state kinetic parameters were measured by using a HPLC-based enzyme assay for product formation, as described in the Experimental Section. Kinetic parameters (±standard error) were determined by fitting the data to the Michaelis–Menten equation by using SigmaPlot; [b] see Scheme 1 for structures.

culture and used to transform chemically competent *E. coli* BL21-(DE3)pLysS, which was screened as described below.

Expression of MatB protein in 96-deep-well microtiter plates: An Eppendorf epMotion liquid handling machine (Hauppauge, NY, USA) was used for liquid transfer steps. Individual colonies of BL21-(DE3)pLysS harboring pET28a–MatB/T207X, pET28a–MatB/M306X, pET28a–MatB or pET28a were used to inoculate wells of a round-bottomed 96-deep-well plate (VWR) containing LB medium (1 mL) supplemented with kanamycin (30 $\mu g\,mL^{-1}$). Culture plates were tightly sealed with AeraSeal breathable film (Research Products International Corp.) and incubated at 37 °C and 350 rpm for 18 h. Aliquots (100 μL) of each culture were transferred to a freshly prepared deep-well-plate containing LB medium (1 mL) supplemented with kanamycin (30 $\mu g\,mL^{-1}$). The freshly inoculated plate was incubated at 37 °C and 350 rpm for 3 h, at which point protein expression was induced with IPTG (1 mM). The plate was incubated for 18 h at 22 °C and 350 rpm. Cells were harvested by centrifugation at 5000 *g* for 10 min and resuspended in Tris-HCl buffer (100 mM, pH 8.0; 250 μL) containing lysozyme (5 $mg\,mL^{-1}$). The plates were then subjected to a single cycle of freeze/thaw, and the cell debris was collected by centrifugation at 5000 *g* for 10 min. An aliquot (50 μL) of each cleared extract was used for the screening reaction.

Colorimetric assay: For the high-throughput screening reaction, cleared extract (50 μL) was added to sodium phosphate buffer (50 mM; 200 μL, pH 7.2) containing $MgCl_2$ (10 mM), ATP (0.5 mM), coenzyme A (0.1 mM), and malonate or an analogue **1b**/**1d** (0.5 mM). The reactions were incubated for up to 4 h, at which point Ellman's reagent (15 μL) was added to each well; (7.8 mM Ellman's reagent stock was prepared by suspending 77.1 mg 5,5′-dithiobis(2-nitrobenzoic acid) in 25 mL of 0.1 M sodium phosphate buffer (pH 7) and readjusting to pH 7, whereupon, all 5,5′-dithiobis(2-nitrobenzoic acid) was dissolved). Upon mixing, the absorbance at 412 nm was measured by using a BioTek Hybrid Synergy 4 plate reader (Winooski, VT, USA).

Expression and purification of WT and mutant MatB: *E. coli* BL21-(DE3)pLysS competent cells were transformed with the suitable plasmid and positive transformants were selected on LB agar supplemented with kanamycin (30 $\mu g\,mL^{-1}$). A single colony was transferred to LB (3 mL) supplemented with kanamycin (30 $\mu g\,mL^{-1}$) and grown at 37 °C and 250 rpm, overnight. The culture was used to inoculate LB media (1 L) supplemented with kanamycin (30 $\mu g\,mL^{-1}$). The culture (1 L) was incubated at 37 °C and 250 rpm to an optical density of 0.6, at which point protein synthesis was induced by the addition of IPTG (final concentration 1 mM). After incubation at 18 °C and 200 rpm for 18 h, cells were collected by centrifugation at 5000 *g* for 20 min, and resuspended in Tris-HCl, pH 8.0 (100 mM; 20 mL) containing NaCl (300 mM), and then lyzed by sonication. Following centrifugation at 10000 *g*, the soluble extract was loaded onto a 1 mL HisTrap HP column (GE Healthcare, Piscataway, NJ, USA) and purified by fast protein liquid chromatography by using the following buffers: wash buffer (20 mM phosphate, pH 7.4, containing 0.5 M NaCl, 20 mM imidazole) and elution buffer (20 mM phosphate, pH 7.4, containing 0.5 M NaCl, 200 mM imidazole). The purified protein was concentrated by using an Amicon Ultra 10000 MWCO centrifugal filter (Millipore Corp., Billerica, MA, USA) and stored as glycerol (10%) stocks at −80 °C. Protein purity was verified by SDS-PAGE. Protein quantification was carried out by using the Bradford protein assay kit from Bio-Rad.

HPLC assay: The in vitro enzyme assays were performed in reaction mixtures (200 μL) containing sodium phosphate (100 mM; pH 7), $MgCl_2$ (2 mM), ATP (0.4 mM), coenzyme A (0.2 mM), malonate or an analogue **1b**–**1d** (0.8 mM) and MatB (1 μg) at 25 °C. Aliquots were removed and quenched with an equal volume of ice-cold methanol, centrifuged at 10000 *g* for 10 min, and cleared supernatants were used for HPLC analysis. A series of linear gradients was developed from 0.1% TFA (solution A) in water to methanol (HPLC grade, solution B) by using the following protocol: 0–32 min, 80% B; 32–35 min, 100% A. The flow-rate was 1 $mL\,min^{-1}$, and the absorbance was monitored at 254 nm by using Pursuit XRs C18 column (250×4.6 mm, Varian, Inc.). A calibration curve with **2b** was constructed to determine concentrations of CoA thiosters in the enzymatic reaction mixtures, from integration of product HPLC peak-areas (see the Supporting Information).

Determination of kinetic parameters: Enzyme assays were carried out in a total volume of 200 μL sodium phosphate (100 mM; pH 7) containing $MgCl_2$ (2 mM) and pure enzyme (1 μg). Kinetic parameters k_{cat} and K_M were determined with **1b**–**1d** as variable substrates, and ATP and coenzyme A concentrations were kept constant (0.4 and 0.2 mM, respectively). Each experiment was performed in triplicate. The incubation time of the assays was 20 min, after which time product formation was still linear with respect to time (see the Supporting Information). Aliquots were removed and quenched with an equal volume of ice-cold methanol, centrifuged at 10000 *g* for 10 min, and cleared supernatants were used for HPLC analysis. The concentration of product formed was determined as described above, and initial velocities were fitted to the Michaelis–Menten equation by using SigmaPlot.

Mass spectrometry analysis: Samples were subjected to negative-ESI LC/MS (Waters BEH C18, 2.1×50 mm, 1.7 m particle). A series of linear gradients was developed from water/1 mM ammonium formate (pH 5.3; solution A) to methanol (solution B) by using the following protocol: 0–10 min, 3–80% B; 10–11 min, 80–95% B; 11–13 min, 95% B; 13–14 min, 9–5% B; 14–17.5 min, 5% B.

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Keywords: directed evolution · enzymes · polyketides · protein engineering · specificity

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