Poly Specific *trans*-Acyltransferase Machinery Revealed *via* Engineered Acyl-CoA Synthetases

Irina Koryakina,[†] John McArthur,[†] Shan Randall,[‡] Matthew M. Draelos,[†] Ewa M. Musiol,[§] David C. Muddiman,[‡] Tilmann Weber,[§] and Gavin J. Williams*,[†]

[†]Department of Chemistry and [‡]W. M. Keck FT-ICR Mass Spectrometry Laboratory, North Carolina State University, Raleigh, North Carolina, United States

[§]Eberhard-Karls-Universität Tübingen, Interfakultäres Institut für Mikrobiologie und Infektionsmedizin, Mikrobiologie/Biotechnologie, Tübingen, Germany

Ⓢ *Supporting Information*

ABSTRACT: Polyketide synthases construct polyketides with diverse structures and biological activities *via* the condensation of extender units and acyl thioesters. Although a growing body of evidence suggests that polyketide synthases might be tolerant to non-natural extender units, *in vitro* and *in vivo* studies aimed at probing and utilizing polyketide synthase specificity are severely limited to only a small number of extender units, owing to the lack of synthetic routes to a broad variety of acyl-CoA extender units. Here, we report the construction of promiscuous malonyl-CoA synthetase variants that can be used to synthesize a broad range of malonyl-CoA extender units substituted at the C2-position, several of which contain handles for chemoselective ligation and are not found in natural biosynthetic systems. We highlighted utility of these enzymes by probing the acyl-CoA specificity of several *trans*-acyltransferases, leading to the unprecedented discovery of poly specificity toward non-natural extender units, several of which are not found in naturally occurring biosynthetic pathways. These results reveal that polyketide biosynthetic machinery might be more tolerant to non-natural substrates than previously established, and that mutant synthetases are valuable tools for probing the specificity of biosynthetic machinery. Our data suggest new synthetic biology strategies for harnessing this promiscuity and enabling the regioselective modification of polyketides.
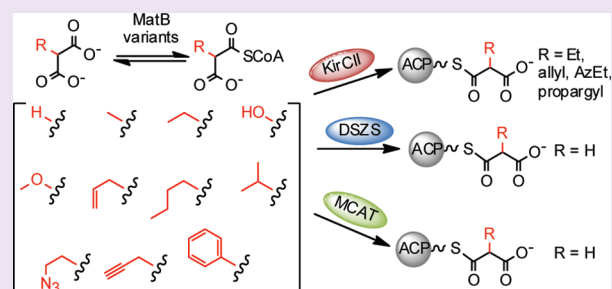
Polyketides are a large class of pharmaceutically relevant natural products with wide-ranging biological activities that include antimicrobial, anticancer, and immunosuppressant.[1] Moreover, polyketides constitute a significant fraction of approved and top-selling drugs, annual sales of which total $20 billion.[2,3] The broad and potent biological activities of polyketides are determined by their chemical structures, the carbon backbones of which are biosynthesized *via* iterative Claisen condensations between activated malonate extender units and acyl thioesters, catalyzed by polyketide synthases (PKSs).[4] A modest variety of malonate extender units substituted at the C2 position are cumulatively available to polyketide biosynthetic pathways,[5,6] although producing organisms typically only possess biosynthetic routes to a small number of extender units, depending on the structures of the corresponding polyketides. The most common PKS extender units include malonyl-Coenzyme A (CoA) (**1**), methylmalonyl-CoA (**2**), and ethylmalonyl-CoA (**3**) (Figure 1). A smaller number of PKSs use specialized extender units that are functionalized directly on standalone acyl carrier proteins (ACPs) and include methoxymalonyl-ACP[7] (**4**), hydroxymalonyl-ACP[8] (**5**), aminomalonyl-ACP[8] (**6**), and allylmalonyl-ACP[9] (**7**) (Figure 1). Additionally, it has recently emerged that several extender units, including chloroethyl,

propyl, hexyl, and isobutylmalonyl-CoA (**8–11**, respectively) (Figure 1) are available to a subset of PKSs *via* the reductive carboxylation of α,β-unsaturated acyl-CoA precursors by crotonyl-CoA carboxylase/reductase (CCR) homologues.[10–12] The selection and incorporation of extender units by PKSs contributes to significant portions of polyketide structure, in addition to the vast structural diversity across the polyketide family, as illustrated by the structures of concanamycin A aglycone (**12**), zwittermicin A (**13**), salinosporamides (**14a–14d**) and the FK506 series (*e.g.*, **15a** and **15b**) (Figure 1). Moreover, variation of extender unit selection and incorporation at specific positions in polyketide backbones has been shown to directly modulate biological activities of polyketides produced by natural PKSs.[9,13]

Even though Nature provides a modest selection of potential PKS extender units, most polyketide-producing organisms provide biosynthetic routes to only a relatively small number of suitable acyl-CoAs. Subsequently, there is a growing body of evidence that PKSs can tolerate non-natural extender units that are not normally provided by the producing host.[14,15] However,

a

1: R = H
2: R = Me
3: R = Et

4: R = OMe
5: R = OH
6: R = NH₂
7: R = allyl

8: R = EtCl
9: R = propyl
10: R = hexyl
11: R = isobutyl

b

12 (concanamycin A aglycone)

13 (Zwittermicin A)

14a : R = EtCl
14b : R = EtBr
14c : R = EtF
14d : R = hexyl

15a (FK506): R = allyl
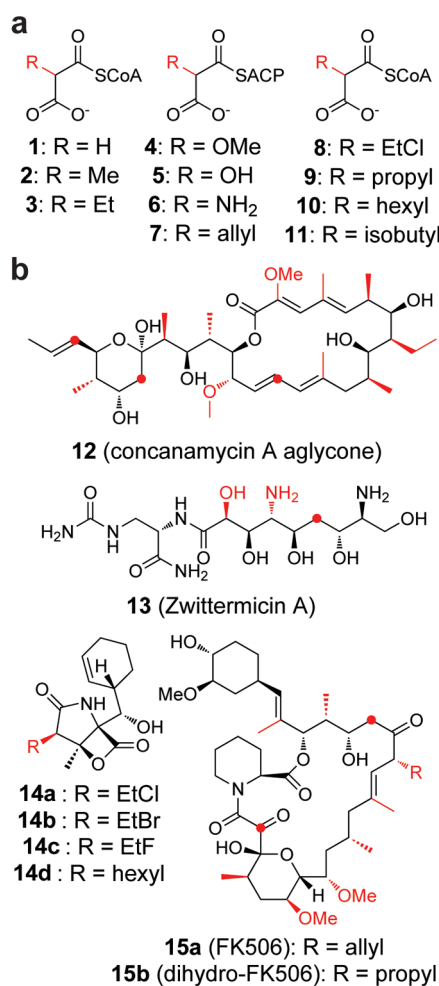**15b** (dihydro-FK506): R = propyl

Figure 1. Variety of naturally occurring PKS extender units and contribution to polyketide structure. (a) Extender units are biosynthesized as acyl-CoAs (**1**−**3**), ACP-linked acyl thioesters (**4**−**7**), or from CRR-mediated reductive carboxylation of α,β-unsaturated acyl-CoA precursors (**8**−**11**). (b) Contribution of extender unit C2-side chains to the structures representative polyketides is highlighted red. Positions derived from C2-H of malonate extender units are shown as red dots.

in vitro and *in vivo* studies aimed at investigating PKS specificity are limited in scope and number,[14,16] owing at least in part to a lack of easily synthesized extender units. We proposed to build on the previously described[15,17] partial promiscuity of the malonyl-CoA synthetase MatB from *Rhizobium trifolii* (Figure 2). This enzyme displays low-level activity toward several alkylmalonates, and we sought to produce a robust biocatalyst for the activation of malonates modified at the C2-position with a broad range of substituents. We postulated that a truly promiscuous synthetase would prove useful for probing the *in vitro* acyl-CoA specificity of PKSs and related biosynthetic machinery. Consequently, sufficiently promiscuous MatB mutants could also form the basis of prototype bacterial strains for *in vivo* extender unit generation and subsequent installation into polyketides.

Here, we report structure-based engineering of MatB to produce variant enzymes capable of synthesizing a broad range of acyl-CoAs, including several that are not produced by the wild-type enzyme. This panel of extender units was used to probe the specificity of *trans*-acyltransferases (*trans*-ATs)

involved in polyketide biosynthesis, revealing unprecedented activities toward non-natural extender moieties. The ability to design acyl-CoA synthetases with new specificities is a first step toward using synthetic biology to create promiscuous *in vitro* and *in vivo* enzymatic systems for generating non-natural polyketide analogues.

■ RESULTS AND DISCUSSION

Structure-Guided Mutagenesis. Recently, following preliminary screening of small MatB saturation mutagenesis libraries against methylmalonate (**17**) and allylmalonate (**20**) (Figure 2b), we reported[18] the identification of two MatB single mutants, T207S and M306I, which displayed improvements in catalytic efficiency toward two extenders, **17** and **20**. In addition, both mutants showed improvements toward ethylmalonate (**18**), which was not originally screened for. Here, in order to expand the potential scope of MatB-based extender unit biosynthesis and to further test our ability to engineer the promiscuity of MatB, we designed a panel of seven additional malonates substituted at the C2-position with diverse functionality (Figure 2b). This panel included members that were reported[15] to be either very poor substrates for the wild-type enzyme (**18**, **21**, **22**; which represent <10% relative reaction rate with malonate **16**) or moderately good substrates (**17**, **20**; 10−20% relative rate with **16**) or were not previously examined (**19**, **23**−**26**). This panel represents broad structural diversity and includes analogues functionalized with chemical handles for downstream chemoselective ligation (**19**, **20**, **26**), which could prove useful for further diversification strategies should these novel extender units become incorporated into polyketides. In addition, three members (**20**, **24**, **25**) represent extender units that are provided to PKSs as ACP-linked substrates in natural biosynthetic systems,[5,9] and acyl-CoA routes to such extenders are not currently known. Moreover, three members (**19**, **23**, **26**) are currently completely unknown to natural biosynthetic systems. Panel members were either commercially available (**16**−**18**, **20**−**24**), prepared from the corresponding commercially available diester (**19**, **25**), or synthesized from diethyl cyclopropyl-1,1-dicarboxylate in the case of **26**. To assess promiscuity of wild-type MatB and the mutants T207S and M306I, kinetic parameters (k_{cat} and apparent K_m) for each analogue were determined (Table 1, Supplementary Figure S1 and Supplementary Table S1) using a robust HPLC assay for acyl-CoA formation.[18] In each case, acyl-CoA product identity was confirmed by LC−MS analysis (Supplementary Table S2). Of the 11 malonates tested (**16**−**26**), only five (**16**−**20**) resulted in formation of the desired acyl-CoA with wild-type MatB (Table 1). Furthermore, the wild-type enzyme clearly favors malonate **16** out of the set and is 2.4-, 93-, 10000-, and 38-fold less efficient (k_{cat}/K_m) with **17**, **18**, **19**, and **20**, respectively. Thus, although wild-type MatB clearly has some capacity to turnover malonate analogues, promiscuity is insufficient to process the entire set. Kinetic parameters for our previously described T207S and M306I were also determined using the entire panel of analogues (Table 1, Supplementary Figure S1 and Supplementary Table S1). As we previously described,[18] both mutants display significant shifts in specificity toward **18** and **20**. Notably, compared to wild-type MatB, the butyl (**22**), methoxy (**25**), and azidoethyl (**26**) analogues are additional substrates for T207S, albeit very poor ones, whereas azidoethyl (**26**) is also a substrate for M306I. Analogues **21** and **23** are non-substrates for both wild-type MatB and the mutants T207S and M306I.
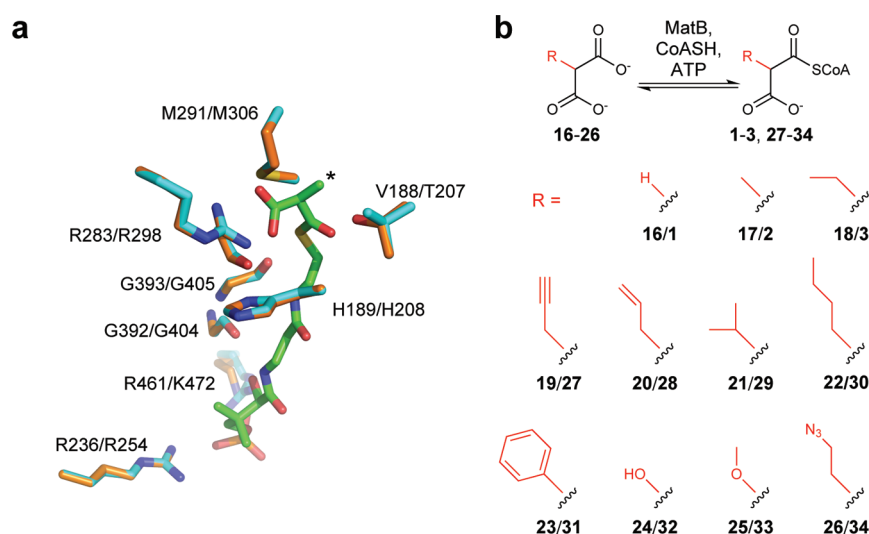
Figure 2. Engineering the acyl-CoA specificity of MatB. (a) Active site architecture of MatB. The *Streptomyces coelicolor* MatB crystal structure (cyan carbons, PDB code 3NYQ) is shown overlaid with a homology model of the *Rhizobium trifolii* MatB (pale green, SWISS-MODEL[38] using 3NYQ as template), illustrating the intimate role T207 and M306 play in extender side-chain specificity. Residue labels are those of *S. coelicolor/R. trifolii*, respectively. Methylmalonyl-CoA is shown with green carbons, and the C2-methyl is indicated with an asterisk. (b) Reactions catalyzed by wild-type and mutant MatB. The natural substrate for MatB is **16**.

Table 1. Specificity Constants (k_{cat}/K_m) (mM^{-1} s^{-1}) of Wild-Type and Mutant MatB with 16−26[a]

enzyme	malonate analogue										
	16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24[b]**	**25**	**26**
WT	50.3	21.0	0.54	0.005	1.3	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
T207G	3.3	16.6	21.4	0.5	49.5	0.03	1.5	0.4	ND[c]	ND[c]	21.1
T207A	16.5	13.3	26.9	0.9	11.3	ND[c]	3.5	ND[c]	ND[c]	ND[c]	5.5
T207S	44.4	35.5	43.6	0.4	40.8	ND[c]	1.4	ND[c]	ND[c]	0.004	2.2
M306V	4.5	0.9	4.2	ND[c]	0.8	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	0.06
M306I	13.4	4.7	29.0	0.01	6.2	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	0.08
T207G/M306V	ND[c]	0.31	30.9	0.02	15.8	4.8	1.5	0.32	ND[c]	ND[c]	58
T207G/M306I	ND[c]	0.78	53.9	0.9	48	8.7	2.7	0.6	ND[c]	0.004	24.5
T207A/M306V	1.7	5.5	14.3	2	2.4	0.2	2.3	ND[c]	ND[c]	0.09	6.8
T207A/M306I	3.6	5.2	26.5	0.1	4.0	0.02	2.5	ND[c]	ND[c]	0.1	22
T207S/M306V	1.6	1.0	22.5	0.02	2.1	ND[c]	0.6	ND[c]	ND[c]	ND[c]	1.2
T207S/M306I	2.8	10.9	219	2.6	92	0.02	1.6	ND[c]	ND[c]	0.08	22.7

[a]Steady state kinetic parameters were determined as described in the Experimental Section, using a fixed concentration of CoA and ATP, and varied concentration of malonate analogue. See Supplementary Figure S1 and Supplementary Table S1 for Michaelis−Menten plots, numerical data, and standard deviations. See Supplementary Table S2 for product characterization by MS analysis. [b]Activity toward **24** was detected, but only under unique assay conditions and only when wild-type MatB was used. [c]Nondetected (ND) refers to an estimated detection limit <0.004 mM^{-1} s^{-1}.

Cumulatively, this data provides evidence that mutation of the MatB active site could provide activity toward malonate analogues that are not detectable substrates for the wild-type enzyme.

To fulfill our goal of generating MatB variants for *in vivo* and *in vitro* production of a broad range of extender units, we set out to further expand the specificity of MatB toward the entire malonate panel (**18**−**26**). Accordingly, we sought to broaden our search by considering other T207X and M306X members that were improved compared to the wild-type enzyme with **18/20** but also less active than T207S and M306I with these substrates. Subsequent re-analysis of our original screening data,[18] DNA sequencing, enzyme purification, and HPLC assay revealed three additional mutants (T207A, T207G, and M306V) that were improved compared to wild-type MatB toward **18/20**. Gratifyingly, kinetic analysis revealed that one of these mutants (T207G) displayed activity with two analogues (**21**, **23**) that were not detectable substrates for either the wild-

type or previously identified single mutants (Table 1, Supplementary Figure S1 and Supplementary Table S1). In contrast, the substrate specificity of T207A and M306V were little changed in comparison to the original mutants T207S and M306I (Table 1, Supplementary Figure S1 and Supplementary Table S1). Not completely satisfied that none of these single mutants were capable of utilizing the entire panel of analogues, nor satisfied with the overall level of catalytic proficiency of these single mutants with some substrates, we next constructed every possible double mutant combination of T207G, T207S, T207A, M306I, and M306V and determined kinetic parameters for the purified enzymes. These double mutants were significantly improved in comparison to the wild-type and single mutant MatB for several reasons. First, both double mutants that contained glycine at position 207 displayed significantly improved activity with **21**, **23**, and **26** (Table 1, Supplementary Figure S1 and Supplementary Table S1). For example, T207G/M306V was 160- and ∼3-fold improved
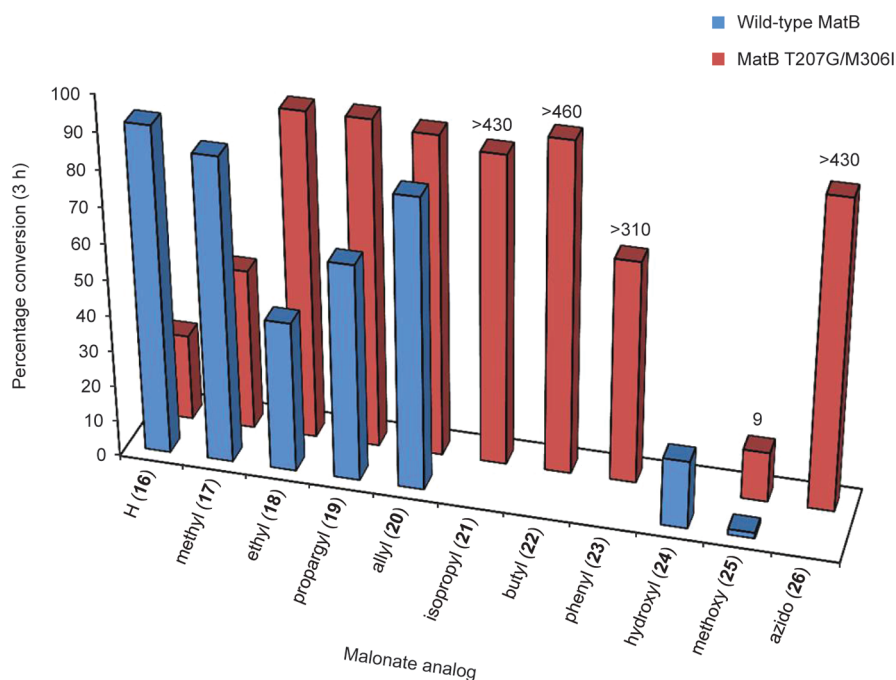
Figure 3. Conversion rates of wild-type MatB and mutant T207G/M306I toward a panel of malonate analogues. Absence of bar indicates no detectable conversion (estimated detection limit 0.2% conversion). Numbers above bars indicate fold-improvement from wild-type enzyme, using an estimated minimal detection limit of 0.2% conversion. See Experimental Section for assay conditions and detection method.

toward **21** and **26**, respectively, in terms of k_{cat}/K_m, compared to the most promiscuous single mutant, T207G (Table 1 and Supplementary Table S1). In addition, T207G/M306I was 290- and 2.5-fold improved toward **21** and **18**, respectively, in terms of k_{cat}/K_m, compared to the parent T207G (Table 1). In contrast, double mutants that contained T207S (the most active position-207 mutant from saturation screening) displayed poorer activity toward **21**, **23**, and **26** compared to T207G/M306I (Table 1, Supplementary Figure S1 and Supplementary Table S1). Gratifyingly though, T207S/M306I was fully 405- and 71-fold improved toward **18** and **20**, respectively, compared to wild-type MatB (Table 1). These data suggest that significant changes in substrate specificity can be obtained *via* relatively subtle alterations to the MatB active site. Accordingly, even relatively low levels of activity (*e.g.,* toward **19** and **23**) may provide suitable starting points for further improvement by directed evolution. Moreover, identification of a set of MatB mutants that can utilize every substrate tested underscores the effectiveness of originally screening MatB mutant libraries against only 1 or 2 substrates. An additional benefit revealed through site-directed recombination is the concomitant reduction in catalytic efficiency with **16** and **17**. In particular, the double mutants T207G/M306V and T207G/M306I do not display detectable activity toward **16** and are fully 114- and 46-fold poorer, in terms of k_{cat}/K_m, toward **17**, compared to the best mutant from our earlier preliminary study, T207S (Table 1 and Supplementary Table S1). This could be highly significant for future *in vivo* applications of the engineered MatB, given that endogenous **16/17** could otherwise be rapidly consumed by MatB, providing little flux through non-natural malonate analogues.

To illustrate the synthetic capabilities of the wild-type and MatB T207G/M306I, a simple end-point HPLC assay was used to compare product conversion after incubation with each malonate analogue. Most notably, even after extended

incubation, wild-type MatB was unable to convert **21–23** and **26** to the corresponding acyl-CoA (Figure 3, Supplementary Figure S2 and Supplementary Table S2). In contrast, MatB T207G/M306I was able to utilize **21–23** and **26** with synthetic conversions ranging from 62% to 92%, in addition to conversion of **16–20** and **25**, with similarly high efficiency. Only **16**, **17**, and **24** were used more efficiently by wild-type MatB. These conversions are in agreement with that expected from the kinetic parameters described above.

Cumulatively, this set of mutagenesis afforded MatB variants that can utilize every malonate analogue tested. The robust synthesis of a broad panel of malonate analogues prompted us to investigate whether these acyl-CoAs could be utilized to discover novel promiscuities displayed *in vitro* by polyketide synthetic systems.

Extender Unit Specificity of *trans*-Acyltransferases. Acyltransferase (AT) domains of typical type I PKSs are responsible for selecting extender units for incorporation into polyketides and are an integral part of each PKS module. A variant of this arrangement involves a freestanding AT enzyme that is physically separated from the PKS and operates in *trans*. Because several such "standalone" *trans*-ATs utilize small molecule acyl-CoAs, versus ACP-linked extender units, they present interesting possibilities for combinatorial biosynthesis of regioselectively modified polyketide analogues.[19,20] To date, however, biochemical studies have only identified three unique *trans*-AT acyl-CoA specificities (**1–3**), while substrate specificity studies of *trans*-ATs and the related transacyltransferases from fatty acid biosynthesis[20–22] that use acyl-CoAs as extender units[23–26] have been limited entirely to the commerically available extender substrates **1/2**, and chemically synthesized **3**. These studies have suggested that *trans*-ATs are highly stringent to their cognate acyl-CoA (*e.g.,* **1**-3). Subsequently, *in vivo* combinatorial biosynthesis efforts involving *trans*-ATs have been restricted to only conservative alterations to
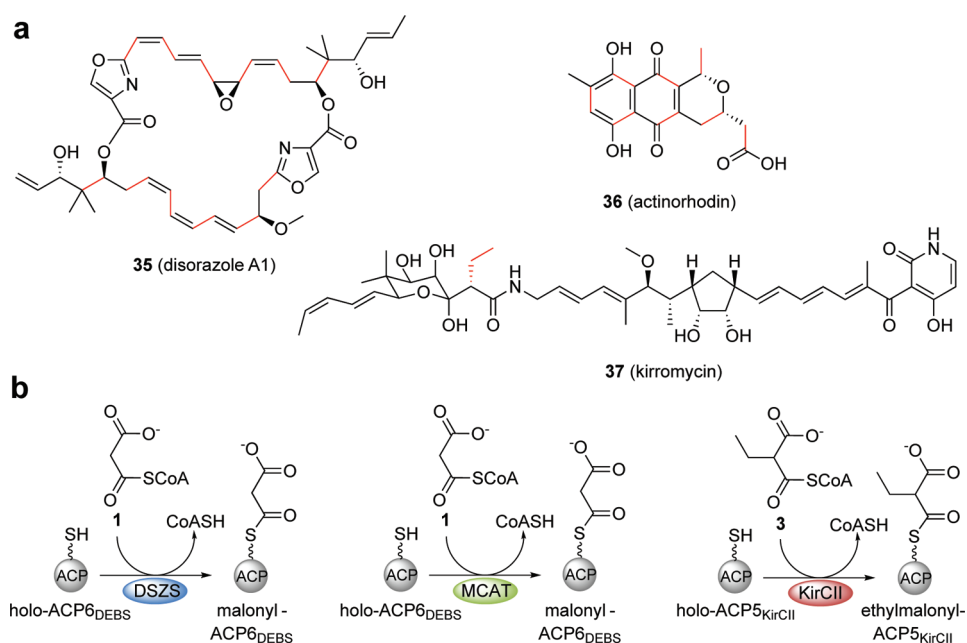
Figure 4. Natural function and assay scheme of DSZS, MCAT, and KirCII. (a) Natural products **35–37** involve the *trans*-ATs DSZS, MCAT, and KirCII, respectively. Contribution of each *trans*-AT to the final product structure is highlighted red. (b) Reaction scheme for each representative *trans*-AT with the corresponding natural acyl-CoA extender unit.

polyketide structure,[20] and the true potential of *trans*-ATs for regioselective polyketide modification is unknown. We therefore used our mutant MatB synthesized acyl-CoA panel to more fully probe the specificity of three representative *trans*-ATs, the malonyl-CoA:ACP transacylase (MCAT) from *Streptomyces coelicolor*,[22] the malonyl-CoA specific DSZS-AT from the disorazole synthase (DSZS),[27] and the ethylmalonyl-CoA specific KirCII from kirromycin biosynthesis[25] (Figure 4a). We hypothesized that KirCII would display some promiscuity toward acyl-CoAs that have C2-substitutions larger than the ethyl side chain of **3** for two reasons: First, both MCAT and DSZS-AT utilize **1** and discriminate significantly against **2** and are therefore unlikely to accept extender units with even larger side chains. Second, in the natural kirromycin producer strain, KirCII does not accept **1**/**2** as substrates, but there is presumably no requirement for KirCII to discriminate against malonate extender units with C2-substituents larger that of **3**.

To test this hypothesis, each *trans*-AT was expressed in *E. coli* as hexahistidine-tagged proteins and purified by nickel-affinity chromatography. For MCAT and DSZS-AT, ACP6 from the 6-deoxyerythronolide B synthase ($ACP6_{DEBS}$) was used as the acceptor substrate,[27] while ACP5 from kirromycin biosynthesis ($ACP5_{Kir}$) was used as the cognate carrier protein for KirCII (Figure 4b).[25] Carrier proteins were converted to their *holo*-ACPs *in vivo* by Sfp-mediated phosphopantetheinylation and subsequently purified by metal chelation chromatography, ensuring removal of Sfp, which is not His₆-tagged. Complete conversion to the *holo* enzyme was confirmed by LC−MS analysis (Supplementary Figure S3). The extender unit specificity of each *trans*-AT was determined using an end-point assay. Briefly, each *trans*-AT was incubated with the corresponding fully activated *holo*-ACP and acyl-CoA (**1–3**, **27–34**). Acylation of the ACPs and percent conversions were determined by Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry-based assay of intact proteins present in the reaction mixtures (Figure 5a,b and Supplementary Table

S3). Negative controls that lacked each *trans*-AT were also analyzed in the same way, in order to identify possible background acylation (Figure 5a,b and Supplementary Table S3). A high level of stringency was chosen for the confirmation of *trans*-AT substrates, whereby acyl-CoAs were considered substrates only when the conversion from *holo*-ACP was completely dependent on the presence of *trans*-AT (Figure 5a,b and Supplementary Table S3). Subsequently, out of 11 extender units tested, only malonyl-CoA **1** led to the identification of the corresponding acylated-ACP (Figure 5a,b and Supplementary Table S3) when MCAT or DSZS were used in these assays. Although the specificity of MCAT or DSZS has not previously been probed beyond **1**/**2**, this stringent malonyl specificity is not completely surprising, given the requirement of these enzymes to at least discriminate against methylmalonyl-CoA. In stark contrast to MCAT and DSZS, KirCII utilized 4 out of 11 acyl-CoA extender units, including the propargyl (**27**), allyl (**28**), and azidoethyl (**34**) analogues (Figure 5a,b), in addition to the established natural substrate (**3**). For each successful reaction (involving acyl-CoAs **1**, **3**, **27**, **28**, and **34**), control reactions that lacked *trans*-AT failed to produce an intact mass corresponding to acyl-transfer (Figure 5a,b, Supplementary Figure S4 and Supplementary Table S3), ruling out self-acylation by the ACP or *trans*-acylation by a contaminant. Although the KirCII-catalyzed acylation of $ACP5_{Kir}$ with **31** was greater than background, we cannot rule out the possibility that **31** does not require the *trans*-AT, given the error of these measurements (Figure 5b). Notably, this discovery stands as the first example of a polyspecific *trans*-AT that can utilize extender unit acyl-CoAs other than **1–3** and demonstrates for the first time *trans*-AT based utilization of extender units not found in natural biosynthetic pathways (**27**, **28**, **34**).

These data suggest that the tolerance of some *trans*-ATs toward non-natural extender units may be considerably higher than previously considered. Presumably, given that KirCII utilizes ethylmalonyl-CoA extender units with the largest C2-
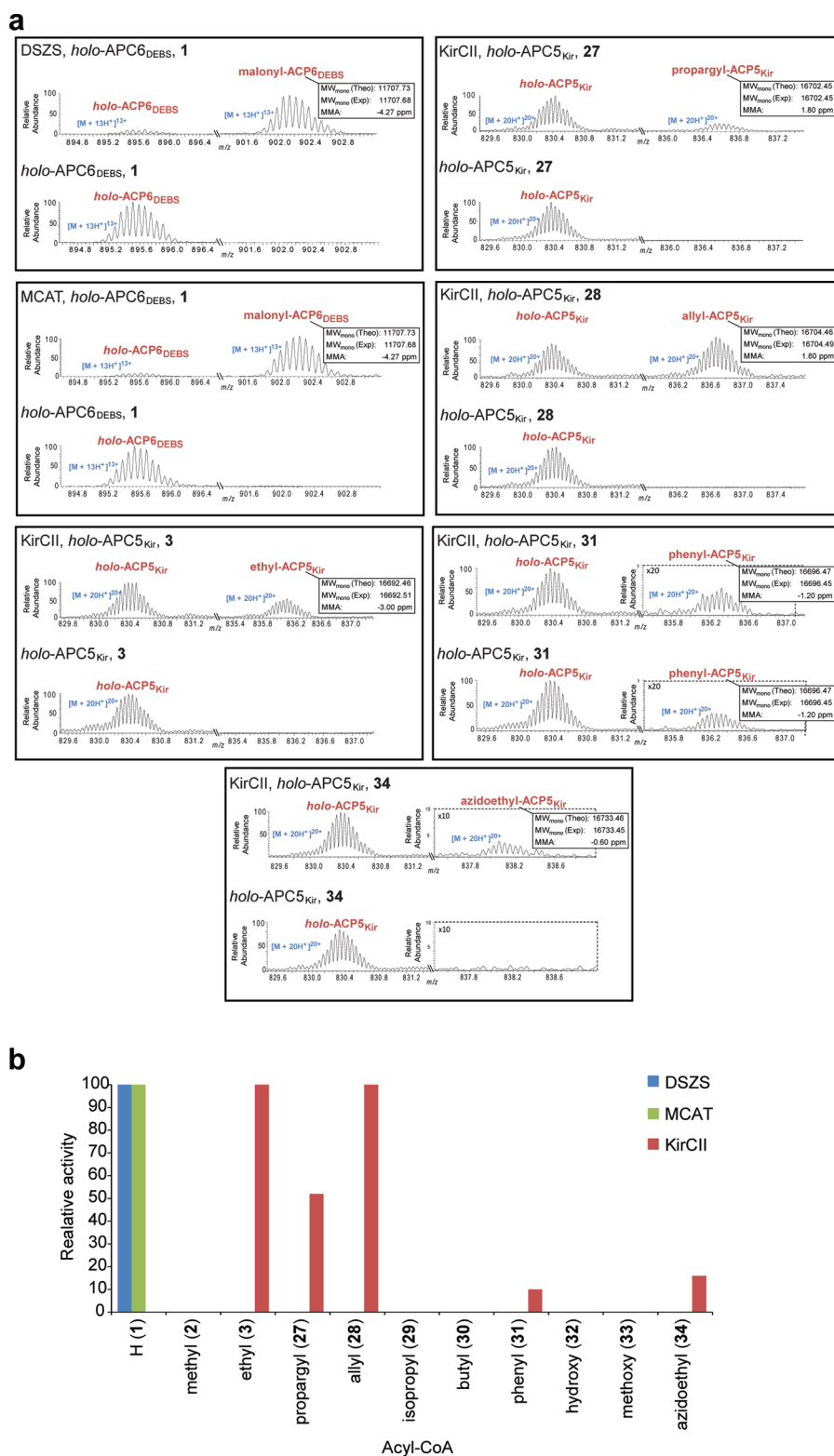
a

b

Figure 5. *In vitro* analysis of MCAT, DSZS and KirCII extender unit specificity. (a) LC-FTICR-MS analysis of successful *trans*-AT catalyzed conversions of *holo*-ACP to acyl-ACP. Each spectrum represents the ^{13}C distribution of the indicated charge state. Each boxed set of spectra shows reactions in the presence (upper) and absence (lower) of each *trans*-AT with the indicated acyl-CoA. Reactions that did not produce the expected mass ion are not shown for brevity. (b) Relative specificity of DSZS, MCAT, and KirCII toward each extender unit. Activity of each wild-type *trans*-AT toward the natural extender unit is set to 100%. Each conversion is corrected using the background acylation rate determined in the absence of *trans*-AT. Acyl-CoAs are considered substrates for the *trans*-AT only when the background rate in the absence of *trans*-AT is zero (see Supplementary Table S3 for complete MS data). The estimated error of these measurements is ±25% of the conversion based on the reaction of KirCII with **27**, which was performed in triplicate. MMA = mass measurement accuracy. See Experimental Section for assay and detection conditions.

substituent available to the producing host, this enzyme does not require discrimination against extender units with larger side chains. Consequently, probing the extender unit specificity of other *trans*-ATs and PKS modules that transfer malonyl-CoA derivatives with C2-substituents that are the largest provided by the host organism might also reveal similar promiscuity. Whereas such candidate *trans*-ATs are currently very rare,[23,28] PKSs that, for example, use the extender ethylmalonyl-CoA[7,29−31] are unlikely to require discrimination against larger extender units (such ATs would nevertheless require discrimination against **1**/**2**). Any such *trans*/*cis*-ATs and the corresponding PKSs might provide valuable promiscuous machinery for future combinatorial biosynthesis efforts.

Implications for *in Vivo* Combinatorial Biosynthesis Strategies. Although acyl-CoA extender units can be chemically synthesized *via* expensive and inefficient synthetic routes, acyl-CoAs are not cell-permeable[32,33] and are therefore ultimately not suitable *in vivo* substrates for polyketide biosynthesis. Yet, natural biosynthetic routes to extender unit acyl-CoAs can be lengthy, sometimes involve several cofactors,[5,6] and have yet to be shown to be suitable targets for protein engineering. Accordingly, beyond the clear demonstration of MatB mutants as important tools for probing *in vitro* PKS specificity, these mutant acyl-CoA synthetases are likely to prove useful for providing extender units for *in vivo* polyketide diversification. Indeed, MatB T207G/M306I shows improvements in both k_{cat} and K_m toward non-natural extender units, and the wild-type MatB-synthesized products are utilized by the erythronolide PKS machinery without the requirement of a suitable promiscuous epimerase.[15] The newly discovered extender unit specificities described here indicate that *trans*-ATs, and possibly by extension *cis*-ATs of the canonical PKSs, could be more versatile with respect to extender unit utilization than previously suggested, and if effectively exploited could offer new strategies to diversify polyketide structure. Thus, the promiscuity of KirCII could be harnessed to produce regioselectively modified kirromycin analogues if endogenous ethylmalonyl-CoA biosynthesis can be knocked out and replaced with our mutant MatB. Moreover, if KirCII displays a level of carrier protein promiscuity similar to that of other biochemically characterized *trans*-ATs,[20,26,27] this enzyme or mutants thereof may provide routes to regioselective modification of other polyketides. In addition, *trans*-ATs, as opposed to their *cis*-acting counterparts, may be good targets for directed evolution studies aimed at further broadening or altering extender unit specificity given their free-standing nature. Indeed, our demonstration that KirCII can utilize extender units modified with handles for chemoselective ligation suggests a potential strategy for screening or selecting novel enzyme activities from large libraries of *trans*-AT variants.

Conclusions. In an effort to overcome the limited specificity of acyl-CoA synthetases, we have used structure-guided saturation mutagenesis and site-directed recombination to improve the promiscuity of the malonyl-CoA synthetase MatB. Notably, the substrate specificity of MatB was considerably expanded by only a small number of amino acid mutations to include five substrates not utilized by the wild-type enzyme (Table 1). Improvements in catalytic efficiency of mutants compared to the wild-type MatB were as high as 14,500-fold (*e.g.*, with **26**), and catalytic efficiencies of the mutant MatBs with several of the analogues were as high as that of the wild-type enzyme with the natural substrate **16** and in some cases were even higher (*e.g.*, T207S/M306I with **18**, **20**).

Further, several newly identified substrates for the engineered MatB mutants contain chemical handles for chemoselective ligation (**19**, **20**, **26**) and are not found in natural extender unit biosynthetic pathways (**19**, **23**, **25**, **26**). Beyond these results, we expect to be able to extend our enzyme engineering strategy to further improve activity of MatB toward analogues that remain relatively poor substrates, such as the phenyl analogue **23**. Additionally, the enzyme engineering strategy described here is expected to overcome the stringent substrate specificity of other naturally occurring acyl-CoA ligases.[34] The creation of promiscuous acyl-CoA synthetases for the chemo-enzymatic synthesis of natural and non-natural extender units represents a considerable advance in our ability to probe the specificity and activity of a broad range of polyketide synthases and related machinery. For example, the availability of a panel of diverse extender units enabled unprecedented poly specificity of a *trans*-AT (KirCII) to be discovered. In summary, our results suggest that mutant synthetases are valuable tools for probing the specificity of polyketide synthase biosynthetic machinery and for future synthetic biology efforts aimed at engineering PKSs for the synthesis of non-natural analogues.

■ EXPERIMENTAL SECTION

General. Unless otherwise stated, all materials and reagents were of the highest grade possible and purchased from Sigma (St. Louis, MO). Isopropyl β-D-thiogalactoside (IPTG) was from Calbiochem. Bacterial strain *Escherichia coli* (*E. coli*) BL21(DE3) pLysS competent cells was from Promega. Primers were ordered from Integrated DNA Technologies. Plasmid pET28a-MatB was as previously described.[18]

MatB Synthetic Assay. *In vitro* enzyme assays were performed in 50 μL of reaction mixture containing 100 mM sodium phosphate (pH 7), MgCl$_2$ (2 mM), ATP (4 mM), coenzyme A (4 mM), malonate or analogue **16−26** (16 mM), and wild-type or mutant MatB (10 μg) at 25 °C. Aliquots were removed after 3 h of incubation, quenched with an equal volume of ice-cold methanol, and centrifuged at 10,000*g* for 10 min and cleared supernatants were used for HPLC analysis on a Varian ProStar HPLC system. A series of linear gradients was developed from 0.1% TFA (A) in water to methanol (HPLC grade, B) using the following protocol: 0−32 min, 80% B; 32−35 min, 100% A. The flow rate was 1 mL/min, and the absorbance was monitored at 254 nm using Pursuit XRs C18 column (250 mm × 4.6 mm, Varian Inc.). The malonate analogue and the acyl-CoA product HPLC peak areas were integrated, and the conversion (%) was calculated as a percent of the total peak area. Product identity was confirmed by LC−MS (Supplementary Table S2).

Mass Spectrometry Analysis of Acyl-CoAs. Samples were subjected to negative-ESI LC−MS on a Thermo TSQ Quantum Discovery MAX connected to a UV−vis diode array detector with a Waters BEH C18, 2.1 mm × 50 mm, 1.7 μm particle column. A series of linear gradients was developed from water/1 mM ammonium formate (pH 5.3) (A) to methanol (B) using the following protocol: 0−10 min, 3−80% B; 10−11 min, 80−95% B; 11−13 min, 95% B; 13−14 min, 95−5% B; 14−17.5 min, 5% B.

trans-AT Assays. DEBS ACP6 and Kir ACP5 were expressed in *E. coli* and purified to homogeneity as completely phosphopantetheinylated *holo*-ACPs using the *B. subtilis* PPTase Sfp. An aliquot of ACP (30 μL) was added to 45 μL of 50 mM sodium phosphate (pH 6.8) (MCAT) or 50 mM sodium phosphate (pH 7.2) (DSZS) or 50 mM Tris-HCI (pH 7.5) containing 50 mM MgCI$_2$ (KirCII) containing 10 μM KirCII or 10 μM DSZS or 10 μM MCAT, and 5 mM acyl-CoA (generated by suitable mutant MatB). Reactions were incubated at 25 °C for 15 min and analyzed by LC-FTICR-MS analysis as described below.

LC-FTICR-MS Analysis of *trans*-AT Reactions. Reaction mixtures were separated using an Eksigent 1D+ nano-LC system utilizing a vented column configuration.[35] Analytical and trap columns were packed in-house with Magic C8 (5 μm particle, 300 Å pore size;

Microm BioResources) stationary phase to approximately 15 and 5 cm, respectively. PicoFrit (75 μm i.d.) and IntegraFrit (100 μm i.d.) capillaries were purchased from New Objective (Woburn, MA). LC solvents were purchased from Burdick and Jackson (Muskegon, MI). Mobile phase A consisted of 98% water, 2% acetonitrile, and 0.2% formic acid, and mobile phase B consisted of 98% acetonitrile, 2% water, and 0.2% formic acid. A 50 ng portion of total protein was injected and desalted at 2 μL/min before switching in-line with the analytical column at a flow rate of 250 nL/min *via* a 10-port valve. The gradient was held at 30% B for 1 min before ramping to 37% B over the next 19 min. The gradient was then adjusted to 95% B in 1 min, holding there for the next 9 min. Finally, the gradient was adjusted to 30% in 1 min and held for an additional 9 min for re-equilibration.

Mass measurements were performed on a 7T LTQ-FT-ICR Ultra (ThermoFisher Scientific) mass spectrometer operating at $50,000_{fwhm}$ resolving power at m/z = 400. Data acquisition was set to one broadband scan event in the ICR cell. Spectra were acquired with an automatic gain control setting of 1×10^6 ions using one microscan with a maximum ionization time of 500 ms. Tube lens voltage was set to 150 V, and the capillary temperature was set to 200 °C.

Determination of monoisotopic masses for intact proteins has been previously described.[36,37] Using a similar approach in this study, monoisotopic masses of identified proteins were determined by overlaying the theoretical isotopic distribution of the most abundant charge state with the experimental isotopic distribution obtained from summed spectra. A Java-based algorithm (Isotopic Pattern Calculator, v1.0) provided as freeware from Pacific Northwest National Laboratory (Richland, WA) was used in conjunction with the molecular formulas of the analytes to generate the theoretical isotopic distributions. Multiply charged spectra were mass transformed to a neutral monoisotopic mass to determine mass measurement accuracy. Although the authors are aware of possible electrospray ionization biases due to differences in hydrophobicity, extracted ion chromatograms corresponding to the *holo*-ACP and acyl-CoA product were generated and integrated for estimating percent conversion using the following formula: $(Area_{acyl\text{-}ACP}/Area_{holo\text{-}ACP} + Area_{acyl\text{-}ACP})$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1–S7, Tables S1–S4, and supplemental methods including site-directed mutagenesis, protein expression and purification of MatB, DSZS, MCAT, DEBS *holo*-ACP6, Kir *holo*-ACP5, and KirCII, determination of MatB kinetic parameters, and synthesis of **19**, **25**, and **26**. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: gavin_williams@ncsu.edu.

Notes
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