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An Evolutionary Model Encompassing Substrate Specificity and Reactivity of Type I Polyketide Synthase Thioesterases

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Bacterial polyketides are a rich source of chemical diversity and pharmaceutical agents. Understanding the biochemical basis for their biosynthesis and the evolutionary driving force leading to this diversity is essential to take advantage of the enzymes as biocatalysts and to access new chemical diversity for drug discovery. Biochemical characterization of the thioesterase (TE) responsible for 6-deoxyerythronolide macrocyclization shows that a small, evolutionarily accessible change to the substrate can increase the chemical diversity of products, including macrodiolide formation. We propose an evolutionary model in which TEs are by nature non-selective for the type of chemistry they catalyze, producing a range of metabolites. As one metabolite becomes essential for improving fitness in a particular environment, the TE evolves to enrich for that corresponding reactivity. This hypothesis is supported by our phylogenetic analysis, showing convergent evolution of macrodiolide-forming TEs.

Modular polyketides, produced by bacteria, represent some of the most chemically rich and biologically potent molecules known. This diverse family of compounds includes clinical antibiotics, anticancer agents, and immunosuppressants. Although highly diverse in structure and function, these compounds are unified by their mechanism of biosynthesis.[1,2] All are produced from simple building blocks in an assembly line-like fashion by multidomain proteins called type I polyketide synthases (PKS). Understanding the detailed mechanism of their biosynthesis is critical to the discovery of new polyketides and to harnessing PKSs to produce new and designer polyketide products.

Phylogenic analysis of type I PKS catalytic domains has shown that substrate specificity or reactivity of the domains often has a primary sequence determinant. For example, the acyltransferase (AT) domains cluster into malonyl-CoA- and methylmalonyl-CoA-specific clades.[3–6] β-ketosynthase (KS) domains from *trans* AT pathways cluster based on the substitution at the α and β carbons of the upstream polyketide intermediates.[7,8] Ketoreductase (KR) domains cluster into type A or type B, based on the facial selectivity for delivery of the hydride from NADPH.[9,10]

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Unique among these PKS domains is the thioesterase (TE). Phylogenic analysis of TEs show that they do not cluster based on substrate specificity or reactivity (Figure 1). Thus, predicting and characterizing TE specificity and reactivity has relied on experimental characterization.[11–13] We characterized the in vitro substrate specificity and reactivity of the TE from the erythromycin biosynthetic pathway (DEBS TE), and herein we show that it is both highly substrate-specific and capable of catalyzing a high diversity of chemistries, including hydrolysis, esterification, macrocyclization, and macrodiolide formation. We propose an evolutionary model using the screening hypothesis[14,15] to account for these observations.

Type I PKS TEs play a key role in the biosynthesis of polyketides, catalyzing release of the completed polyketide chain from the PKS to which it is covalently linked.[16] This step is essential for turnover of the biosynthesis machinery and provides an opportunity to increase the chemical diversity and complexity of the polyketide product. Type I PKS TEs can catalyze hydrolysis of the acyl-PKS intermediate, generating free acids as seen in ambruticin[17] and gephyronic acid[18] biosynthesis, transesterification with an intermolecular alcohol to generate esters as has been observed in vitro,[19] macrocyclization with intramolecular alcohols to generate macrolactones as seen in erythromycin[20] and epothilone[21,22] biosynthesis, or dimerization with a second equivalent of acyl-PKS to generate macrodiolides as is predicted in elaiophylin[23] and disorazol[24] biosynthesis.

The best-studied type I PKS TE is the TE from the erythromycin biosynthetic pathway, DEBS TE. In vitro biochemical characterization of DEBS TE has shown that macrocyclization activity is highly substrate-selective, with hydrolytic activity predominating in virtually all substrates investigated. For example, a small change in oxidation state or stereochemistry of the two substrates known to undergo macrocyclization completely abolishes macrocycle formation.[11,25,26] The TE from the pikromycin biosynthetic pathway(PIK TE) demonstrates similarly high substrate specificity for macrocyclization, suggesting that this is a general phenomenon of type I PKS TEs.[13,26]

In our efforts to investigate the origin of substrate selectivity for macrocyclization, we synthesized substrates that were biosynthetically related to a known DEBS TE macrocyclization-competent substrate, **3** (Scheme 1). Compound **3** possessed key design features we wished to retain in the panel of substrates used for this study. These included the replacement of the ethyl group from **1** with a benzyl group, as this was known to be effectively processed by DEBS TE and provided an easily detectable chromophore,[25,27] the amide linkage, which facilitates synthesis, and the *N*-acetylcysteamine (NAC) activation of the carboxylate, which has been extensively used

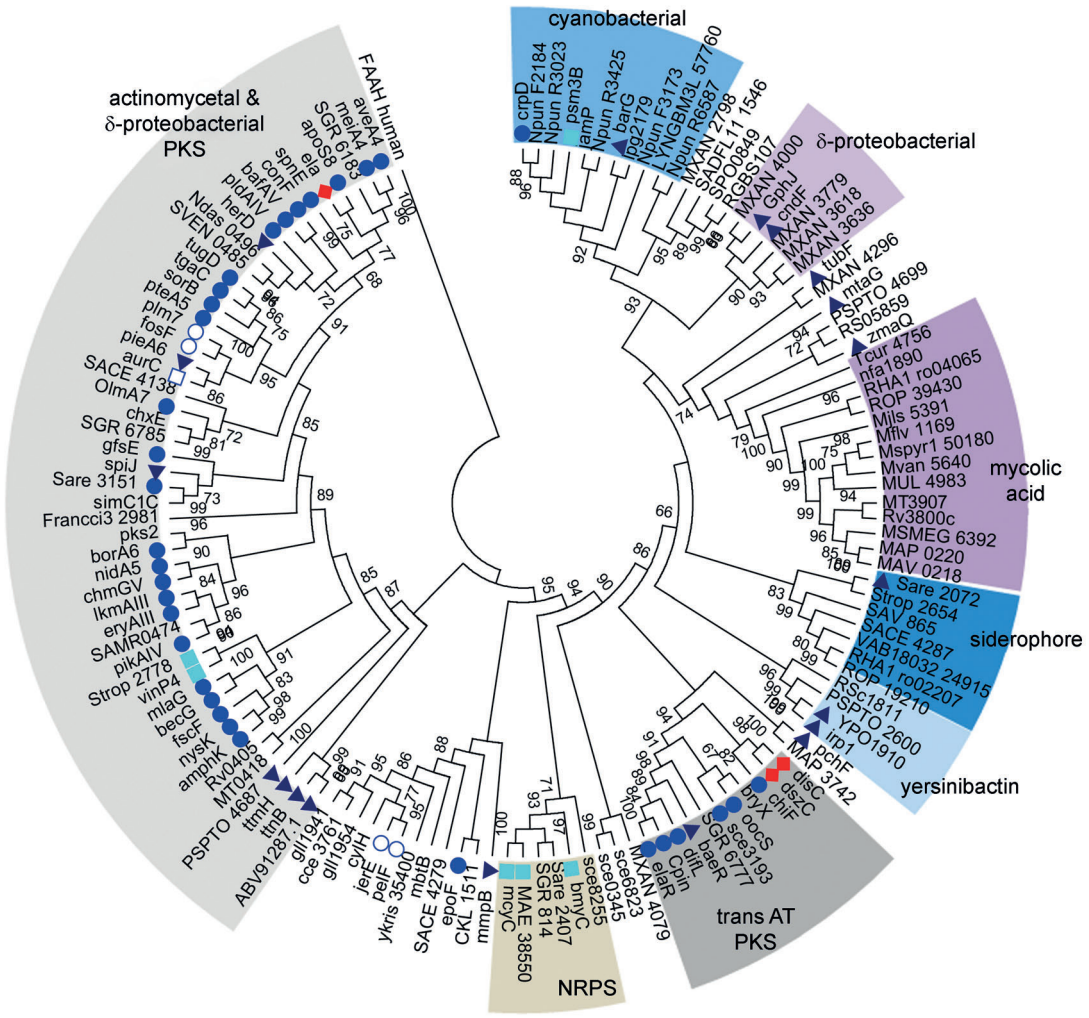
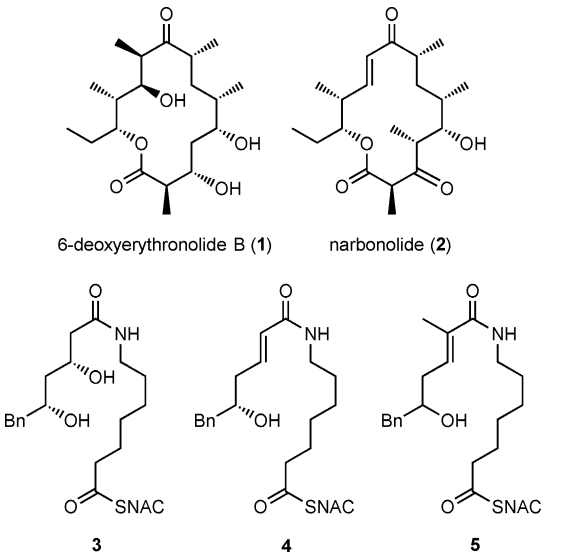


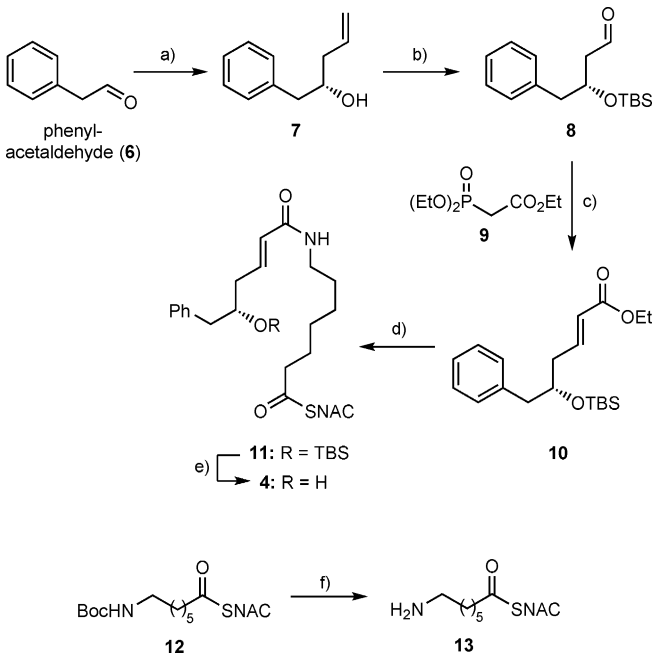
Figure 1. Phylogenetic analysis of 138 TE domains shows that they do not cluster based on substrate specificity or function. Pathway type and origin appear to be greater determinants for clustering of TEs. Sequences and phylogenetic methodology can be found in the Supporting Information. ●: macrolactone, ○: δ-lactone, □: pyrone, ◆: macrodiolide, ■: macrolactam, ▲: acid.

with DEBS TE[11,25,26,28–31] and mimics the cysteamine and C terminus of β-alanine from the native substrate's phosphopantetheinyl group.[30,32] Compound **4** could be envisaged as arising from a biosynthetic gain of dehydratase (DH) function, resulting in conversion of the β-hydroxy functionality of **3** into an α,β-unsaturated carbonyl. The relationship between **3** and **4** is similar to what is seen for 6-deoxyerythronolide B (**1**) and narbonolide (**2**), which are generated by the related TEs DEBS TE and PIK TE, respectively. The enantiomer, *ent*-**4**, was synthesized as a negative control compound, as no D-configured alcohol has been shown to be macrocyclized in vitro or in vivo by DEBS TE.[25,27,33–36] Substrate **5** was envisioned as being related to **4** through modulation of the specificity of the acyltransferase domain, from malonyl-CoA-specific to methylmalonyl-CoA-specific. This panel of substrates provided compounds that could be expected to arise through the evolution of PKS pathways, thus enabling us to provide some of the first insights into the relationship between substrate selectivity and PKS pathway evolution.

Synthesis of **4** and *ent*-**4** was accomplished in eight linear steps (Scheme 2). An asymmetric Brown allylation[37,38] of phenylacetaldehyde (**6**) set the configuration of the stereogenic carbon, furnishing the homoallylic alcohols **7** and *ent*-**7** in moderate yields. These were silylated, and the allylic double bonds were oxidized to yield aldehydes **8** and *ent*-**8**. Horner–Wadsworth–Emmons coupling between **8** and phosphonate **9** was accomplished in moderate yield, furnishing the *E*-α,β-unsaturated esters **10** and *ent*-**10**. Subsequent hydrolysis of the ethyl ester gave the carboxylic acid, which was treated with EDC and coupled with free amine **13** to yield the silylated NAC-thioester substrates **11** and *ent*-**11** in good yield. Deprotection in an acetonitrile solution of HF/pyridine[25] completed the synthesis of enantio-enriched substrates **4** and *ent*-**4** (Scheme 2 and Scheme S2 in the Supporting Information). Substrate **5** was generated in racemic form through an analogous route, starting with the racemic form of aldehyde **8** (Scheme S3). Wittig coupling with methyl 2-(triphenylphosphoranylidene)propionate, gave the methyl substituted α,β-un-



Scheme 1. Substrate **3** is known to undergo macrocyclization with DEBS TE. Substrates **4**, *ent*-**4**, and racemic **5** were designed based on the relationship between 6-deoxyerythronolide B (**1**) and narbonolide **2** to evaluate the effect of evolutionarily accessible substrate modifications on the function of the DEBS TE.



Scheme 2. Synthesis of L-enantio-enriched substrate **4**: a) (−)-Ipc$_2$BAllyl, −100 °C, 90% *ee*, 65%; b) TBSCl, imidazole; $K_3Fe(CN)_6$, K_2OsO_4; $Pb(OAc)_4$, 70% over three steps; c) **9**, KOH, 63%; d) NaOH; **13**, EDC, DMAP, Et_3N, 72% over two steps; e) HF, pyridine, 64%; f) HCl/Dioxane, 100%. The D-enantio-enriched substrate *ent*-**4** was prepared in identical fashion by using (+)-Ipc$_2$BAllyl.

saturated ester, which was converted into the NAC-thioester substrate following analogous chemistry to **4** and *ent*-**4**.

To evaluate the ability of DEBS TE to macrolactonize **4**, *ent*-**4**, and **5**, purified recombinant DEBS TE[30] (15 μM) was treated with each NAC-thioester substrate (2.5 mM) in 50 mM phosphate buffer (pH 7.4) with DMSO (10% *v*/*v*). After 18 h, the reactions were quenched and analyzed for product formation by LC/MS. The results obtained for **4** and *ent*-**4** were consistent with the stereochemical hypothesis indicating that L-config-

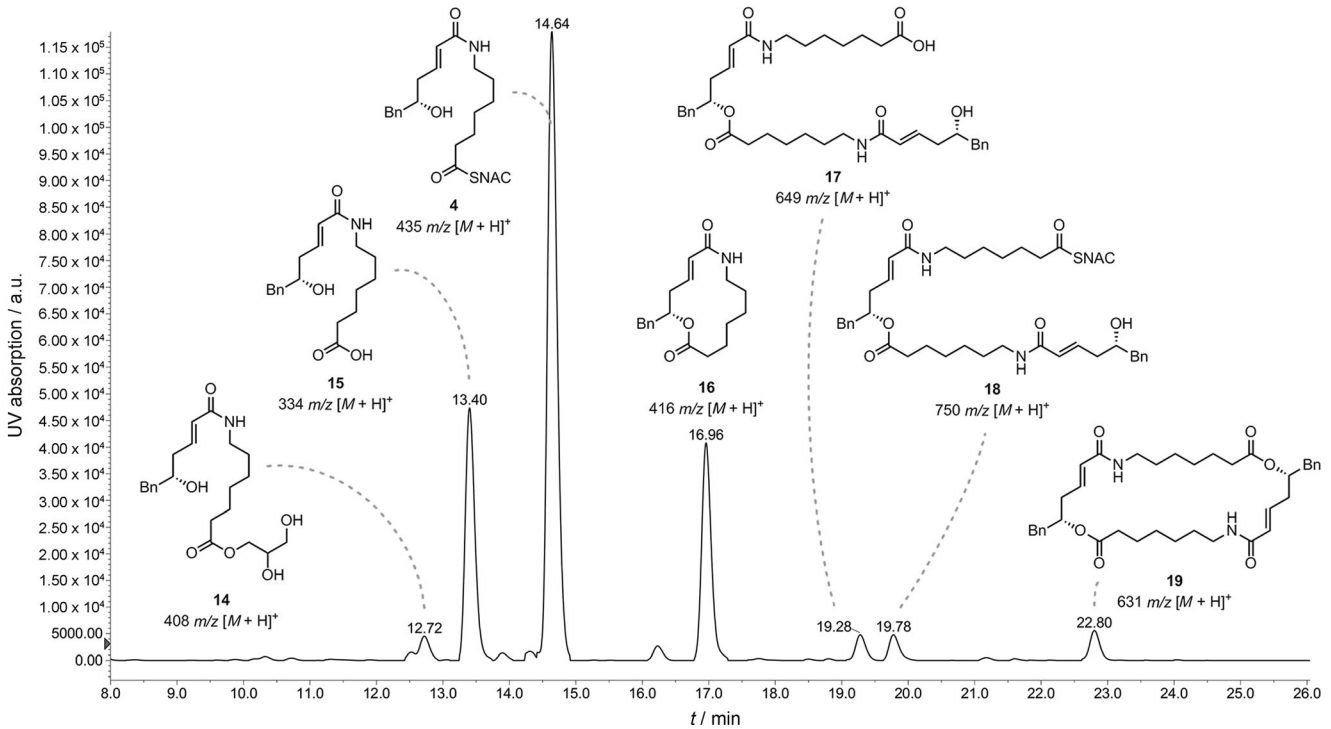


Figure 2. LCMS analysis of the enzymatic reaction of **4** with DEBS TE. The UV (254 nm) trace between 8 and 26 min is shown. Major and minor products are indicated on each trace with the observed *m*/*z* values.

ured alcohols were necessary for macrocyclization (Figures 2 and S4). The L-configured alcohol **4** was clearly converted into the 14-member ring macrolactone **16**, whereas the D-configured alcohol *ent*-**4**, did not appear to be converted. Although trace **16** can be observed from prolonged incubation of *ent*-**4** with DEBS TE (Figure S4), the level of **16** formation was consistent with conversion of trace **4** found in *ent*-**4** into macrocycle (*ent*-**4** *ee* 90%, see the Supporting Information). Incubation of **4** and *ent*-**4** with DEBS TE D169A, which is folded but catalytically inactive,[30] showed no consumption of the substrates or product formation (Figures S7–S12). Macrolactone **16** obtained from the treatment of **4** with wild type DEBS TE was confirmed by MS and ^{1}H NMR analysis. Kinetic characterization of DEBS TE activity with **4** and *ent*-**4** displayed Michaelis–Menten kinetics with k_{cat} values of 0.228 ± 0.015 min^{-1} and 0.114 ± 0.008 min^{-1}, respectively, K_M values of 2.8 ± 0.4 mM and 1.3 ± 0.2 mM, respectively and k_{cat}/K_M values of 1.4 ± 0.3 M^{-1}s^{-1} and 1.5 ± 0.3 M^{-1}s^{-1}, respectively (Figures S13 and S14). These data are consistent with the observed catalytic efficiencies of in vitro characterized modular polyketide TEs.[11–13,25,26,28–31,39,40] For example DEBS TE processed **3** with a specificity constant of 1.2 ± 0.5 M^{-1}s^{-1}, which is in excellent agreement with our data.

Analysis of the reaction products for **4** also demonstrated that the C_2- symmetrical, head-to-tail dimerized macrodiolide product **19** ([*M*+H]$^+$ 631 *m/z*) was also formed. In addition, the glycerolysis adduct **14** ([*M*+H]$^+$ 408 *m/z*), hydrolysis product **15** ([*M*+H]$^+$ 334 *m/z*), the linear dimer *seco*-acid **17** ([*M*+H]$^+$ 648 *m/z*), and linear dimer NAC-thioester **18** ([*M*+H]$^+$ 750 *m/z*) were also observed (Figure 2). The structures of the dimeric compounds were consistent with ^{1}H NMR and MS/MS data collected on these compounds (Supporting Information). The formation of the macrodiolide and linear dimer structures is unprecedented for the DEBS TE, though TEs from the biosynthesis of elaiophylin[23] and disorazol[24] are proposed to catalyze this chemistry.

The mechanism for related non-ribosomal peptide synthetase TE-mediated dimer formation has been investigated in vitro.[41–43] Enterobactin TE, which trimerizes a 2,3-dihydroxybenzoyl-L-serinyl monomer and macrocyclizes it to generate a macrotriolide has been biochemically characterized. By using top-down mass spectrometry, a mutant of this TE was shown to accumulate monomeric and dimeric acyl-enzyme intermediates. It was postulated that the dimer was formed by the seryl side chain alcohol of the monomer-TE attacking an activated thioester of a second equivalent of monomer.[41] The formation of **18** in the current study strongly suggests that a second equivalent of monomer attacks the electrophilic monomeric TE acyl-enzyme intermediate, generating the dimeric NAC-thioester **18**. This type of mechanism was also proposed for the formation of the macrocyclic dimeric non-ribosomal peptide, gramicidin.[43] We thus propose that in our assay, formation of the macrodiolide **19** and the dimeric free acid **17** occurs by loading of **18** onto the TE to generate a dimeric acyl-enzyme intermediate, which can undergo macrocyclization and hydrolysis, respectively.

This mechanism requires an exogenous nucleophile (a second equivalent of monomer) to attack the TE acyl-enzyme intermediate, releasing a linear ester. This type of transesterification activity has been seen with glycerol and type I PKS TEs,[19] as well as with the TE for the fungal pathway responsible for zearalenone biosynthesis (Zea TE).[44] In the case of the Zea TE, a wide range of simple exogenous nucleophiles were shown to be capable of accessing the acyl-enzyme intermediate. We thus hypothesized that if dimer formation was occurring through exogenous nucleophile attack on the acyl-enzyme intermediate by a second equivalent of monomer, then other exogenous nucleophiles should also be able to attack the acyl-enzyme intermediate. We therefore investigated the ability of **4** to undergo DEBS TE-catalyzed transesterification with exogenous alcohols. A panel of alcohols were incubated with **4**, generating the corresponding ester adducts (Table 1). The formation of the ester products was consistent with our proposed mechanism for dimer formation, further supporting this mechanism. Comparison of the relative conversion to the transesterification product showed that primary alcohols were converted most effectively into esters as compared to secondary and tertiary alcohols. As was seen with the transesterification catalyzed by the fungal Zea TE, butan-1-ol was the most competent alcohol substrate for DEBS TE.[44] This similar substrate specificity in two diverse TEs suggests that transesterification could be a broadly conserved activity for PKS TEs; however, further examples are needed to test this hypothesis.

Table 1. Relative velocities for DEBS TE-mediated crosscoupling with exogenous *O*-nucleophiles.[a]

Nucleophile	Relative conversion	Nucleophile	Relative conversion	Nucleophile
butan-1-ol	1.00 ± 0.08	pentan-3-ol	0.26 ± 0.02	butan-1-ol
propan-1-ol	0.85 ± 0.16	pentan-1-ol	0.22 ± 0.08	propan-1-ol
methanol	0.38 ± 0.03	hexan-1-ol	0.17 ± 0.05	methanol
ethanol	0.36 ± 0.04	*t*-butanol	0.14 ± 0.01	ethanol
cyclohexanol	0.34 ± 0.02	propan-2-ol	0.12 ± 0.01	cyclohexanol

[a] For 15 μM DEBS TE with 2.5 mM **4** in 50 mM phosphate buffer (pH 7.4) at 23 °C, with DMSO (10%, *v/v*) and 100 μM nucleophile. Negative controls lacking DEBS TE showed no reactivity.

Incubation of DEBS TE with **5**, the methyl-substituted derivative, led to formation of the hydrolysis product with no detectable macrocycle. Kinetic characterization of the enzymatic activity fit the Michaelis–Menten model, providing a k_{cat} value of 0.20 ± 0.01 min^{-1} and a K_M value of 0.75 ± 0.13 mM (Figure S15), consistent with type I PKS TE-catalyzed chemistries.[11–13,25,26,28–31,39,40] The addition of the α-methyl group substantially increases the allylic 1,3-strain[45] across the amide bond, limiting access to the *S-trans* configuration required for macrocyclization. The observed lack of macrocyclization with **5** thus suggests that DEBS TE was unable to overcome intrinsic unfavorability of the reactive conformation of the substrate.

The screening hypothesis suggests that long-term evolutionary selection favors pathways that support chemical diversity and can be readily "screened" in the face of new environ-

ments.[14,15] In part, the diversity in a multistep pathway is dependent on substrate tolerance of late-stage enzymes. This tolerance permits plasticity in the upstream components of the pathway and fitness-based screening of the newly released compounds. In addition, the off-loading enzymes should be able to process diverse substrates and introduce substantial new chemical diversity to amplify the new compounds accessible from upstream modifications.

Our results, in light of the screening hypothesis, suggest a model for the evolution of type I polyketide chemical diversity in the context of TE-mediated off-loading. We propose that ancestral TEs were able to catalyze a variety of chemistries including hydrolysis, esterification, amidation, macrocyclization, and macrodiolide formation. This range of reactivity expands the chemical diversity accessible from a PKS pathway. As one metabolite, such as a macrodiolide, becomes essential for improving fitness in a particular environment, the TE evolves under pressure to enrich for the corresponding reactivity. This is supported by the phylogenic analysis, which is consistent with convergent evolution of macrodiolide-forming activity. We hypothesize that the ability to access this diversity of reactivity is retained as TE evolves to become selective for a particular product. Based on this hypothesis, we predict that small changes to the substrate can unlock access to this ancestral activity. This has been observed in the in vitro characterization of the DEBS TE domain, where subtle structural changes lead to dramatic changes in reactivity, from hydrolysis, to macrocycle formation, to macrodiolide formation.

Our fitness-based model is sufficient to account for the observed experimental TE activity results in the literature. It can also be used predictively. For example, polyketide natural products have been isolated with functional groups derived from TE-mediated hydrolysis, macrolactonization, macrolactamization, and dimerization. However, no transesterification-derived polyketide products have been isolated. Having observed a butan-1-ol kinetic preference in two diverse TEs, we predict that a butan-1-ol ester would likely be natively produced by a yet unidentified PKS pathway. Furthermore, as the presence of butan-1-ol is required for this activity, we predict that it will be observed in a native producer of butanol, such as the genus *Clostridium*, when at least three of the forty sequenced genomes (*Clostridium kluyveri* DSM 555, *Clostridium botulinum* A2 BoNT, and *C. botulinum* H04402 065) encode a TE[46] and possess a butanol dehydrogenase A (*bdhA*) orthologue.[47]

In summary, we have used phylogenic analysis and in vitro biochemical characterization to develop and support an evolutionary model in which the plasticity of TEs increases long-term fitness through the retained ability to generate and access chemical diversity. We biochemically showed with recombinant DEBS TE that small, evolutionarily accessible changes to the substrate can lead to multiple different chemistries, including hydrolysis, macrocyclization, dimerization, macrodiolide formation, and transesterification.

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Keywords: macrodiolide · macrolactone · screening hypothesis · substrate selectivity · thioesterase

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