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Synthetic biology for heterologous expression and engineering of fungal polyketide synthases

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Polyketide synthases (PKSs) are essential enzymatic systems that produce a wide range of natural products. Their biosynthetic features offer significant opportunities to create a broad range of polyketides for pharmaceutical, agricultural, and biotechnology applications. In this highlight article, we provide a brief overview of key aspects of PKS biosynthesis, their classification in bacteria and fungi, and the evolutionary mechanisms driving their diversification. After outlining the fundamentals of PKSs, we discuss two key design principles for fungal PKS engineering: host selection and engineering strategies. For host selection, we revisit the state of the art in heterologous expression in yeast and filamentous fungi. We then explore strategies for modifying fungal PKS activity through starter unit selection, point mutations, and domain swaps. Finally, we offer perspectives on future directions for fungal PKS engineering, highlighting the importance of exploring new hosts and integrating advanced computational tools.

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1. Polyketide synthases

Polyketides are a major group of natural products with a broad range of biological activities. Outstanding examples of polyketides of bacterial origin are erythromycin (antibiotic), candicidin (antifungal), and mithramycin (antibiotic), while lovastatin (cholesterol-lowering drug), griseofulvin (antifungal), brefeldin (antibiotic), and strobilurin (fungicide) are produced by fungi.[1,2] Polyketides are biosynthesized by enzymatic complexes called polyketide synthases (PKSs). The genes coding for PKSs are often associated with other genes encoding enzymes that tailor their products, regulate their expression, and transport them. These groups of genes are known as biosynthetic gene clusters (BGCs).

PKSs use mostly acetyl-CoA (acetyl coenzyme A), malonyl-CoA and other acyl-CoA precursors to assemble complex organic molecules *via* a thiotemplate mechanism. Broadly speaking, the PKS catalytic cycle is initiated when the acyltransferase (AT) domain transfers a starter substrate, typically acetyl-CoA, to an acyl carrier protein (ACP). The ACP, which requires post-translational activation by a phosphopantetheinyl transferase (PPTase) to become functional, shuttles the tethered intermediate between catalytic domains. The chain elongation proceeds by adding the extender unit malonyl-CoA, which undergoes a decarboxylation reaction catalyzed by the ketosynthase (KS) domain, forming a carbon–carbon bond through a Claisen condensation. The β-keto intermediate can be further modified through the action of β-processing domains. The ketoreductase (KR) domain reduces the carbonyl group on the β-keto intermediate to a hydroxyl group. Subsequently, the dehydratase (DH) domain dehydrates the obtained hydroxyl group to form a carbon–carbon double bond, and the enoyl reductase (ER) domain reduces the alkene to yield a fully saturated chain (Fig. 1). All type I PKSs, which form multimodular complexes, also include the pseudo-KR (ψKR) domain,[3] that structurally resembles the KR domain, lacks catalytic activity, and plays structural or regulatory roles in PKS function. Furthermore, the polyketide chain can be methylated. The origin of α-methyl groups is a key difference between bacterial and fungal polyketides. In fungi, the *C*-methyltransferase (*C*-MeT) domains catalyze the methyl transfer from the cofactor *S*-adenosylmethionine (SAM) to the α-carbon of β-ketoacyl substrates. Although bacteria usually use methyl-malonyl-CoA

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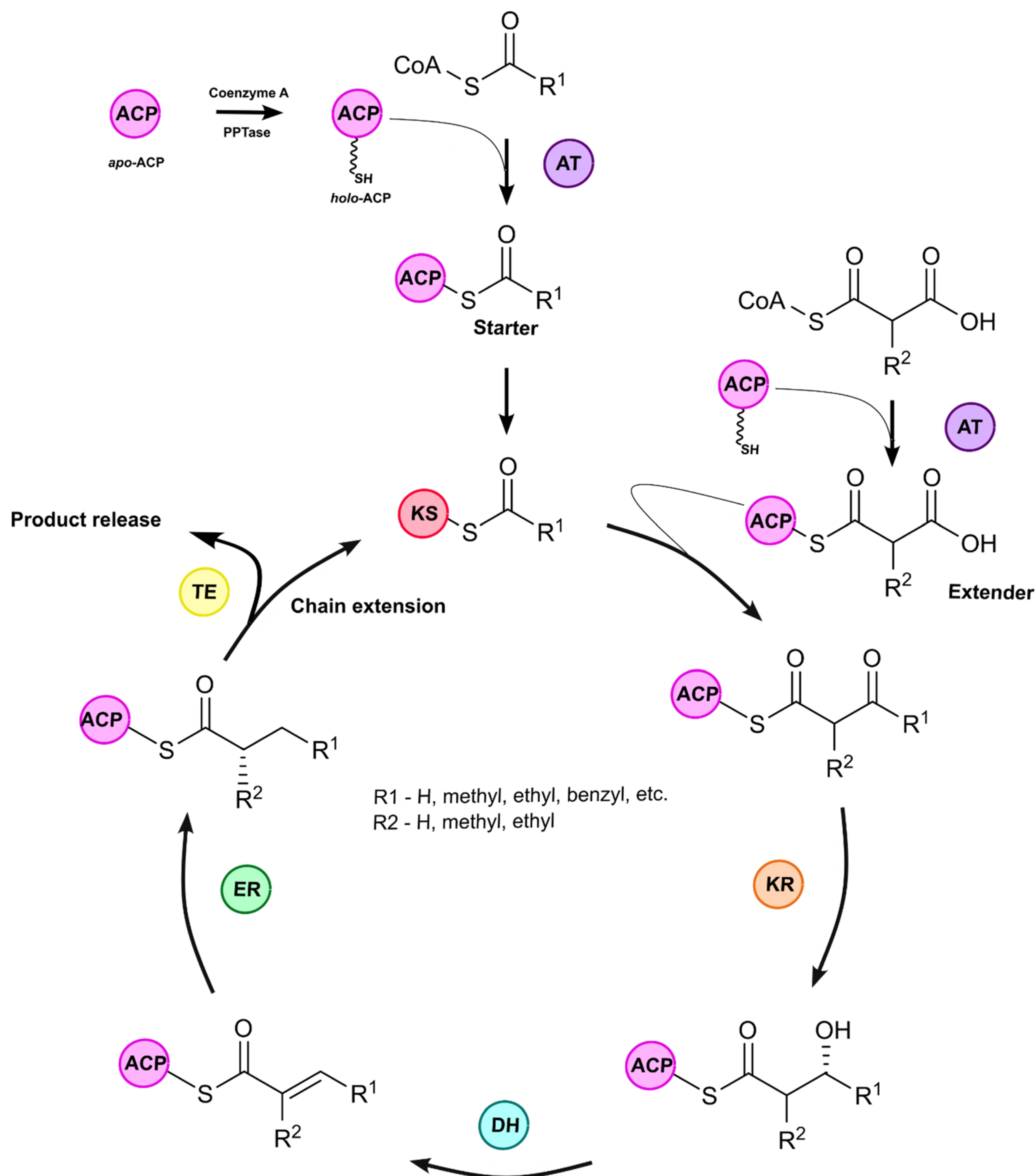

Fig. 1 Biosynthetic mechanism of PKSs. Acetyltransferase (AT), ketosynthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), thioesterase (TE), acyl carrier protein (ACP), phosphopantetheinyl transferase (PPTase), phosphopantetheinyl arm (squiggle –SH) (adapted and modified from Cano-Prieto *et al.*[6]).

as a precursor, SAM-dependent *C*-MeT domains are also found in bacterial *trans*-AT PKSs.[4] Once the polyketide chain reaches its desired length, the termination step is usually catalyzed by thioesterase (TE) or reductase (R) domains, cleaving the chain from the enzyme.[5]

2. Frequency and classification of PKSs

PKSs are found in the bacterial and fungal kingdoms, in some unicellular eukaryotes like amoeba, haptophyta, and apicomplexans, and in some animals.[7–12] PKSs are classified into three

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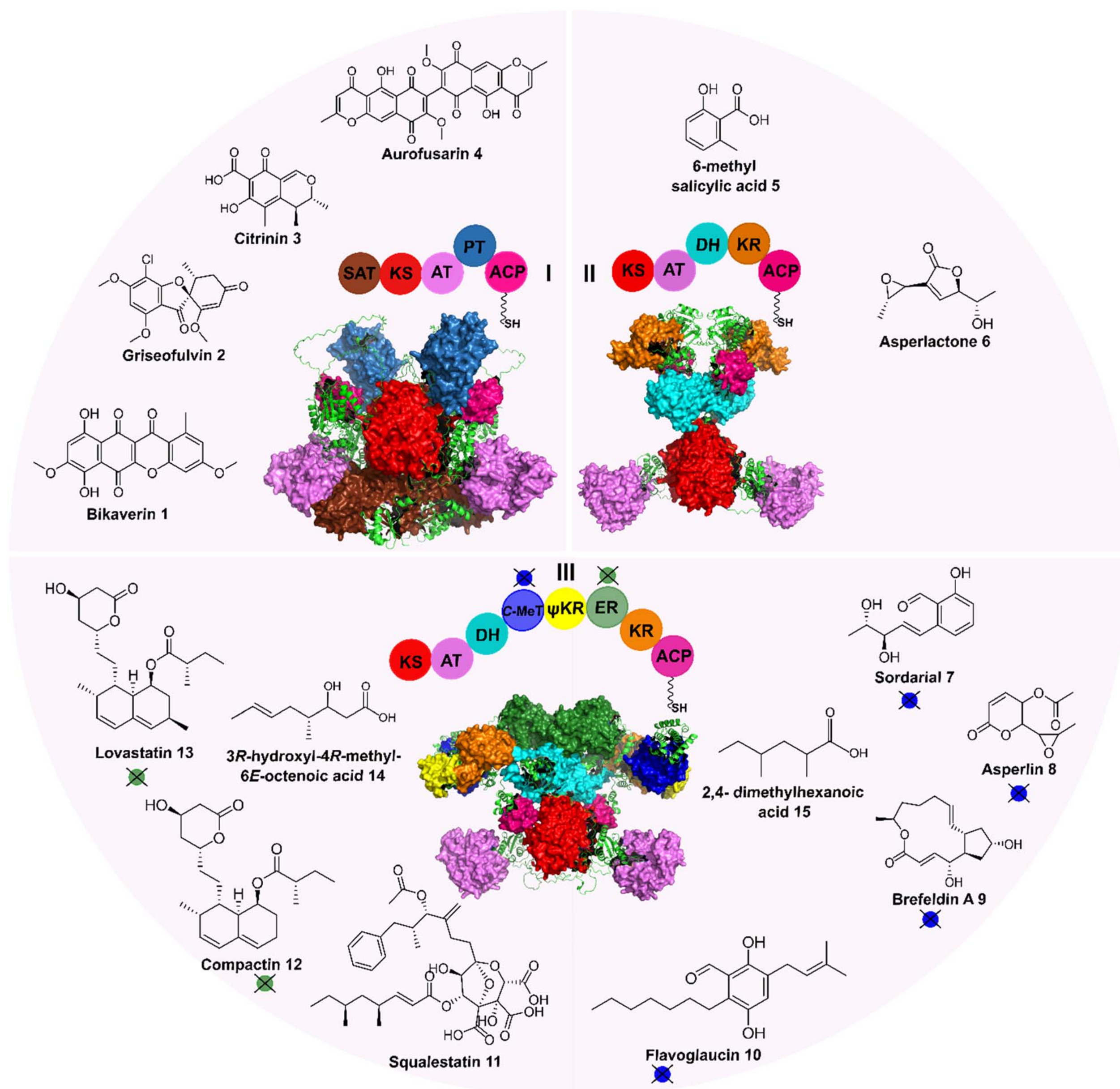

Fig. 2 Major types of fungal iPKSs and product examples. (I) SAT domain containing PKSs, (II) partially reducing PKSs, (III) highly reducing PKSs. Compounds **11**, **14**, and **15** are the products of PKSs with fully functional domains. Compounds **12** and **13** are produced by PKSs with an inactive *cis*-ER domain, whereas compounds **7–10** are from PKSs with a nonfunctional *C*-MeT domain. The blue and green circles with cross indicate inactive *C*-MeT and *cis*-ER domains respectively. PKSs structures were generated using AlphaFold 3.[44]

types based on their functional domains and structural organization. Type I PKSs are large megaenzymes and are further subdivided into modular and iterative PKS (iPKS). Modular PKSs possess discrete modules for each elongation cycle, whereas iPKSs rely on repeated use of a single module to construct the polyketide backbone. Modular PKSs can be classified further into *cis*-AT and *trans*-AT PKSs. In *cis*-AT PKSs, the AT domains are embedded within each module, whereas in *trans*-AT PKSs, the AT domain is separately encoded as a standalone protein.[13] Type II PKSs consist of standalone monofunctional enzymes that assemble into multienzyme complexes with iterative catalytic activities. Lastly, type III PKSs form homodimers of twin KS domains. Despite some mechanistic parallels with other PKS types, type III PKSs are evolutionarily distinct and characteristically ACP-independent; plant chalcone synthases are a well-known example of this type.[14]

Type I multimodular PKSs are often formed by a set of modules in which each performs a single extension–reduction catalytic cycle (*i.e.*, their modules do not iterate). Therefore, the final product usually corresponds to the number of modules in the synthase, a trait known as co-linearity. Type I multimodular PKSs are the most common class in bacteria,[15] with canonical examples being the PKSs directing the biosynthesis of 6-deoxyerythronolide B in *Saccharopolyspora erythraea* and PKS

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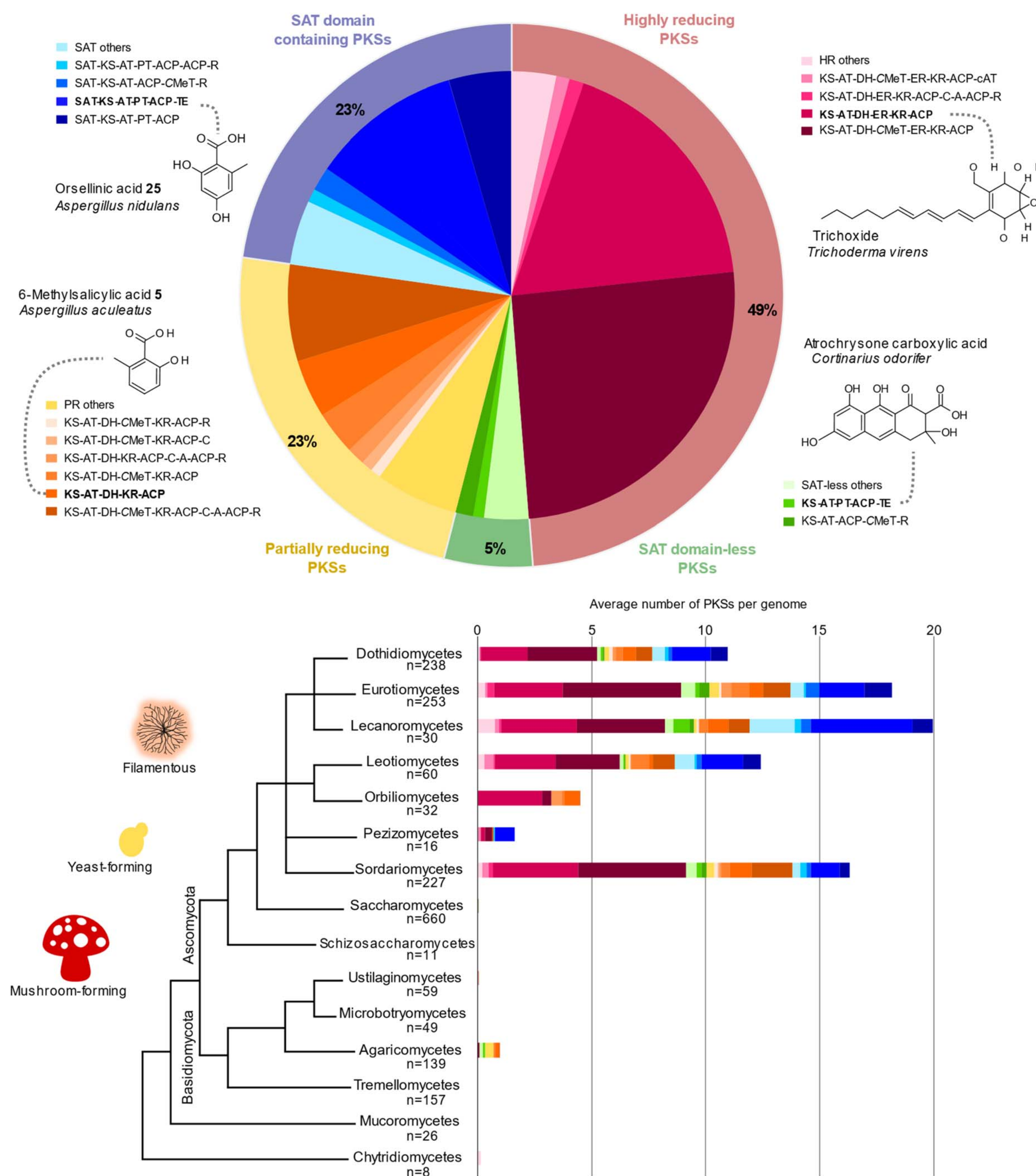

Fig. 3 Meta-analysis of PKSs encoded in fungal genomes. Top: Distribution of different types of PKSs and corresponding domain organization across all fungal PKSs. Bottom: Distribution of PKSs among the fungal kingdom, highlighting the types of PKSs in each class. Partially reducing (PR), highly reducing (HR), SAT domain-containing (SAT), SAT domain-less (SAT-less) PKSs. The *n* represents the number of genomes in each class. The number of PKSs has been calculated as the average number of PKSs per genome in each class. SI file 1 contains the data used for this analysis. The PKS distribution data were obtained from 839 fungal genomes available in GenBank. The genomes were annotated using AUGUSTUS[45] and antiSMASH 7.0 (fungal version).[46] PKS domain organization was extracted from the antiSMASH output files using custom code available at https://github.com/WeMakeMolecules/fun-git/blob/main/AS_domain_parser_folder.pl. The resulting data were used to generate this figure and are available as SI.

producing 6,8*a*-seco-6,8*a*-deoxy-5-oxoavermectin aglycon in *Streptomyces avermitilis*.[16,17]

Almost all fungal PKSs, apart from the small number of type III enzymes are type I iPKS.[18] Based on the presence of reducing domains, the iPKSs can be categorized into three main types: highly reducing PKS (HR-PKS), partially reducing PKS (PR-PKS) and non-reducing PKS (NR-PKS).[5] The HR-PKSs domain architecture includes all necessary domains to fully modify a β-keto group to a saturated acyl chain, namely KR, DH, and ER domain. Thus, the minimal domain organization for an HR-PKS

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includes KS-AT-DH-ER-KR-ACP, typically with a *trans*-acting offloading system, such as a TE domain. All HR-PKS systems also contain a *C*-methyltransferase (*C*-MeT) domain, or a residual domain structure that yields α-methylated moieties.[19] Although the *C*-MeT domains may be catalytically inactive, it is nevertheless conserved in fungal HR-PKSs, similar to the inactive residual *C*-MeT domain found in vertebrate fatty acid synthase (FAS).[20] Some examples of HR-PKS include LovB involved in the biosynthesis of lovastatin **13** in *Aspergillus terreus*, Phpks1 involved in squalestatin **11** biosynthesis in *Phoma* sp. MF5453, MlcA involved in compactin **12** biosynthesis in *Penicillium citrinum*, SrdA involved in sordariol **7** biosynthesis in *Neurospora crassa*, AlnA involved in asperlin **8** biosynthesis in *A. nidulans*, Bref-PKS involved in brefeldin A **9** biosynthesis in *Eupenicillium brefeldianum*, FogA involved in flavoglaucin **10** biosynthesis in *A. ruber*, SimG involved in 3*R*-hydroxyl-4*R*-methyl-6*E*-octenoic acid **14** biosynthesis in *T. inflatum*, AzaB involved in 2,4-dimethylhexanoic acid **15** biosynthesis in *A. niger* (Fig. 2).[21–29] In PR-PKSs, the ER domain is missing, and their products may feature double bonds, hydroxyl, and ketone groups. Examples of PR-PKSs include 6-methylsalicylic acid **5** synthase (MSAS), involved in the biosynthesis of 6-methylsalicylic acid (6-MSA) in *A. aculeatus* and Aomsas, which is involved in the biosynthesis of asperlactone **6** *via* **5** as an intermediate in *A. westerdijkiae* (Fig. 2).[30,31]

NR-PKSs include a subgroup of PKSs in which the starter unit loading requires a dedicated N-terminal starter unit: ACP transacylase (SAT) domain (Fig. 2). Fungal NR-PKSs usually harbor a SAT domain and a product template (PT) domain but lack the β-processing domains responsible for reductive or dehydrative steps. The SAT domain initiates polyketide biosynthesis, and the PT domain catalyzes ring cyclization.[32,33] Although the PT domain is evolutionarily related to the DH domain, they do not catalyze dehydration; instead, they direct ring closure by exploiting the nucleophilicity of the reactive enol intermediate derived from the unreduced β-keto species.[34] Examples of NR-PKSs include those involved in the biosynthesis of citrinin **3** (CitS) in *Monascus ruber*, aurofusarin **4** (PKS12) in *Fusarium graminearum*, bikaverin **1** (Bik1) in *F. fujikuroi*, and griseofulvin **2** (GsfA) in *P. aethiopicum*.[35–38] Although the essentiality of the SAT domain to start the catalytic cycle of NR-PKSs used to be considered a paradigm for this class, the recent discovery of inactive SAT domains with a glycine in place of the typical catalytic cysteine and SAT-less NR-PKS from Basidomycetes represent exceptions to this rule.[39,40]

The iPKSs are also widely distributed among bacteria but are less abundant than multimodular type I PKSs.[41] Some bacterial PKSs are semi-iterative; in these cases, at least one of the modules in a multi-modular PKS performs several rounds of extension. For instance, in the biosynthesis of aureothin, the first module catalyzes two condensation steps before the second module becomes competent.[42]

Given the extensive characterization of bacterial BGCs, their PKS diversity and distribution have been studied in depth.[12] In contrast, fungal BGCs, despite being simpler in structure due to monocistronic gene organization, remain less explored, largely due to the relative complexity of fungal genomes and underrepresentation in sequencing efforts.[6] For this piece, we conducted a meta-analysis using publicly available fungal genomes to find if trends in abundance and diversity of PKS subclasses exist across the fungal kingdom. For a more in-depth study of the biosynthetic diversity of the fungal kingdom we strongly recommend the work by Robey *et al.*[43] We surveyed about 1965 fungal genomes from many different groups, including both Ascomycota and Basidiomycota. We found that 49% of the fungal genomes analyzed harbor genes encoding HR-PKSs (Fig. 3). Among these, about half are predicted to harbor the *C*-MeT domain for methylation, making highly reducing-methylated PKSs the most abundant type of synthase. We also observed that PKSs of any type are most likely to be found in classes rich in filamentous species such as Dothidiomycetes, Eurotiomycetes, Sordariomycetes, Lecanoromycetes, Leotiomycetes, and Orbiliomycetes. Meanwhile, zero or very few PKSs can be found in classes rich in unicellular fungi, like the Saccharomycetes and Schizosaccharomycetes. Basidiomycetes in general are poor in PKSs, except for the Agaricomycetes, which are rich in SAT-less NR-PKSs.[39]

3. Evolutionary mechanisms for diversification of PKSs

It is widely accepted that type I PKSs are closely related to animal FAS.[47] While it is impossible to precisely reconstruct the events that led to the emergence of PKSs, it has been established that four key genetic mechanisms – gene duplication, horizontal gene transfer, gene conversion, and recombination – play a role in PKS evolution.[48] Much of what is currently known about the PKSs evolution comes from bacteria, where extensive genomic and functional data are available. In contrast, the evolutionary trajectories of fungal PKSs remain less well understood due to the relative complexity and availability of less fungal genomes.

In contrast to bacterial multimodular PKSs, the majority of fungal PKSs are monomodular, highly reducing, and iterative systems. While fungal iPKSs include HR-, PR-, and NR-PKSs, this discussion focuses on HR-PKSs, which represent approximately 50% of all fungal iPKSs in sequenced genomes (Fig. 3). HR-PKSs are programmed to control chain length, methylation, reduction, and stereochemistry, thereby delivering one or a limited set of products.[49] The programming of these HR-PKSs is primarily attributed to finely tuned kinetics and substrate specificities across multiple catalytic domains. Thus, intrinsic selectivity and catalytic competence are central to HR-PKS programming.[50] The mechanistic constraints of monomodularity suggest that the evolution of fungal PKS systems can be primarily explained by gene duplication, divergence, and loss.[51] Here, these evolutionary processes alter catalytic efficiency, domain composition, and specificity, ultimately determining the product outcomes of the newly evolved PKS.

The process leading to new iPKSs is not as studied as multimodular synthases; however, some aspects of the programming of these synthases reveal some evolutionary insights. During the biosynthetic cycle of HR-PKSs, each domain

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catalyzes its designated reaction with substrate preferences such as chain length, functionalization pattern, and stereochemistry. This built-in substrate specificity determines which domains can process the intermediate at any given iteration.[49] In parallel to substrate specificity, catalytic efficiency defines the PKS output. Each domain's catalytic rate defines the likelihood of participating in each iteration. A key example highlighting the role of catalytic efficiency in iPKS programming is the competition between KR and *C*-MeT domains, which has been extensively analyzed by Cox *et al.*[3] In their groundbreaking work Cox and team established that the differences in the catalytic rate of KR can influence whether and where methylation occurs. Thus, the timing of methylation is determined by how effectively the KR domain competes for the substrate with the *C*-MeT domain, rather than by intrinsic differences in the *C*-MeT domain itself.[3,50,52]

Beyond chain tailoring, the onset of product off-loading determines the structure of the final product. Chain termination in fungal HR-PKSs often occurs when a required domain fails to act on the substrate. In bacterial type II systems, KS domains have been implicated in chain-length control,[53] but there is limited evidence that the KS domains in HR-PKSs play a similar role. Instead, it is often the interplay of domain activity (or inactivity) that dictates when the polyketide is complete and to be offloaded by *cis* or *trans* acting enzymes.[50]

Despite advancements in structural biology and biochemical characterization of HR-PKSs, the evolutionary and mechanistic details governing intrinsic and extrinsic selectivities remain only partially understood. Further studies are needed to determine how various domains coordinate with one another, how skipped reduction or methylation steps are kinetically dictated, and what truly dictates chain-length. The lack of a mechanistic and evolutionary framework for type I iterative PKSs makes it difficult to predict product outcomes from primary sequence data alone.[50]

4. Design principles

4.1 Host selection and heterologous expression

Fungal polyketides are a valuable source for the development of drugs and agrochemicals, with a large, untapped potential. However, accessing this potential can be challenging. Typically, the expression of some fungal PKSs and their BGCs is tightly regulated and triggered by specific and almost always unknown environmental conditions.[54] Therefore, some of the strategies to identify the product of a given BGC is to activate its expression, *e.g.* by using different culture media and growth conditions.[54] Methods such as "One Strain Many Compounds" (OSMAC) have been used to activate fungal BGCs.[55] Other strategies include the use of co-cultivation approaches to activate the BGCs and the use of chemical elicitors such as epigenetic modulators, which have been shown to increase the number of metabolites by 35%.[56–58]

However, these methods can be expensive, time-consuming, and often result in low efficiency activation of novel BGCs. Moreover, the titer of polyketides resulting from the activation of BGC might be insufficient for their characterization.[59] Heterologous expression is an alternative method to systematically identify and characterize the products of PKS-encoding BGCs. The genetic engineering tools for heterologous expression of PKSs and other fungal natural products have been well described previously,[60] thus here we focused more on the selection criteria for heterologous hosts for fungal polyketide production.

Heterologous expression bypasses native regulation by transferring the BGCs to well-known heterologous hosts with characterized, controllable and robust expression systems such as the bacterial host *Streptomyces coelicolor*,[61] fungal hosts *Saccharomyces cerevisiae* or Aspergilli, facilitating the production of PKSs.[48] Examples of heterologous expression of PKSs in these hosts are listed in Table 1. Another key advantage of heterologous expression is that the native host is only required to obtain the sequences of PKS-coding and accessory genes, which can then be chemically synthesized and used, avoiding the limitations of PCR-based or direct capture methods.[62]

S. cerevisiae and Aspergilli are the predominant heterologous hosts for the production of fungal polyketides due to their well-known physiologies and availability of tools for their genetic manipulation.[62,63] *S. cerevisiae* offers numerous advantages, such as fast engineering cycles, scalability, availability of high-copy plasmids, and low metabolic background. Nevertheless, prior to expressing PKSs in *S. cerevisiae*, it is necessary to perform genetic modifications such as the integration of a PPTase for the activation of the PKSs, commonly a copy of the *npgA* gene from *A. nidulans*.[64] Further metabolic modifications can be required to redirect fluxes of acetyl- and malonyl-CoA towards the PKSs, which might be required for robust production.[65] Even with these modifications, heterologous production of PKSs in *S. cerevisiae* often results in low titers when expressing HR-PKSs,[63] which may require time-consuming troubleshooting experiments, including the use of different promoters, high-copy plasmids, reducing incubation temperatures, and culture scale-up to obtain sufficient amounts of polyketides for downstream application.[66,67] These are some reasons why Aspergilli are often preferred over *S. cerevisiae* for the heterologous production of HR-PKSs, as seen in numerous studies (Table 1).

Aspergilli are prolific producers of a wide range of polyketides (Fig. 3) and offer numerous advantages. For example, their intron-processing machinery is often compatible with heterologous PKSs coding sequences, their robust metabolism includes essential accessory enzymes such as a PPTase, and their precursor pools are readily available for the expression of heterologous PKSs and downstream tailoring.[59] In addition, Aspergilli can readily grow in sustainable feedstocks such as xylose,[68] while *S. cerevisiae* prefers glucose and can only grow in xylose after metabolic engineering approaches.[69] Nevertheless, most Aspergilli (except the domesticated *A. oryzae*) possess more complex chemical backgrounds, as the genus belongs to a class with the highest average number of BGCs in the fungal kingdom[59] (Fig. 3). Despite the deletion of BGCs in some Aspergilli to reduce metabolite background, their metabolic profile remains complex and hinders the detection and purification of exogenous polyketides.[70] Moreover, the native

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Table 1 Notable examples of heterologously expressed type I fungal iPKSs products from 2010 to 2025

Molecule	Type	Domain organization	Native host	Heterologous host	References
Atranorin	NR	SAT-KS-AT-PT-ACP-TE	*Stereocaulon alpinum*	*Ascochyta rabiei*	86
Bostrycoidin **16**	NR	SAT-KS-MAT-PT-ACP-TR	*Fusarium solani*	*S. cerevisiae*	87
2,4-Dihydroxy-3,5, 6-trimethylbenzaldehyde, 6-ethyl-2,4-dihydroxy-3, 5-dimethylbenzaldehyde	NR	SAT-KS-AT-PT-ACP-*C*-MeT-R	*A. nidulans*	*A. niger*	88
Lecanoric acid **17**	NR	SAT-KS-MAT-PT-ACP-TE	*Pseudevernia furfuracea* (Perfume lichen)	*S. cerevisiae*	89
3-Methylorcinaldehyde	NR	SAT-KS-AT-PT-ACP-*C*-MeT-R	*Acremonium strictum*	*A. oryzae*, *Trichoderma reesei*	90 and 91
Naptho-pyrone Ywa1	NR	SAT-KS-AT-PT-ACP	*Paecilomyces variotii*	*A. nidulans*	92
Orsellinic acid **25**	NR	SAT-KS-AT-PT-ACP-TE	Many fungal species and lichenized fungi	*A. niger*, *A. oryzae*	93 and 94
Topopyrones	NR	SAT-KS-AT-PT-ACP-TE	*Phoma* sp. BAUA2861	*A. oryzae*	95
3,5-Dimethyl orsellinic acid, nor-toralactone, and 6-MSA **5**	NR and PR	SAT-KS-AT-PT-ACP-TE, SAT-KS-AT-PT-ACP-TE, KS-AT-DH-KR-ACP	Aspergilli and *Penicillium*	*A. nidulans*, *P. crustosum*	96
facms0007	PR	KS-AT-DH-KR-ACP	*A. terreus*	*A. nidulans*	97
facms0015	PR	KS_AT-DH-*C*-MeT-KR-ACP	*A. wentii*	*A. nidulans*	97
Pyrrolocin A	PR (PKS-NRPS hybrid)	KS-AT-DH-*C*-MeT-KR-ACP-C-A-T-R	Endophytic fungus, designated strain NRRL 50135	*F. heterosporum*	98
Asperfuranone	NR and HR	SAT-KS_AT-PT-ACP-*C*-MeT-R, KS-AT-DH-*C*-MeT-ER-KR-ACP	*A. terreus*	*A. nidulans*	99
Beauveriolides	HR (PKS-NRPS hybrid)	KS-AT-DH-*C*-MeT-ER-KR-ACP	*Cordyceps militaris*	*A. nidulans*	100
Betaenone	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP-R	*Phoma betae*	*A. oryzae*	101
Cordypyrone A and B	HR	KS-AT-DH-KR-ACP	*C. militaris*	*A. nidulans*	102
Cyclopiazonic acid **18**	HR (PKS-NRPS hybrid)	KS-AT-DH-KR-ACP-C-A-T-R	*A. flavus*	*F. heterosporum*	98
facms0003	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP	*A. aculeatus*	*A. nidulans*	97
facms0010	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP	*A. wentii*	*A. nidulans*	97
4-Hydroxy pyridones	HR (PKS-NRPS hybrid)	KS-AT-DH-*C*-MeT-ER-KR-ACP-C-A-T-DKC	*Tolypocladium* sp. 49Y	*A. oryzae*	103
Ilicicolin H and J	HR (PKS-NRPS hybrid)	KS-AT-DH-*C*-MeT-ER^0-KR-ACP-C-A-T-DKC	*Neonectria* sp. DH2	*A. nidulans*	104
Lovastatin **13** and FR901512	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP, KS-AT-DH-ER-KR-ACP	*A. terreus* and *Xylaria grammica*	*S. cerevisiae*	105
Monacolin K	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP	*Monascus pilosus*	*A. oryzae*	106
Pretenellin A **19**	HR (PKS-NRPS hybrid)	KS-AT-DH-*C*-MeT-ER^0-KR-ACP-C-A-T-DKC	*Beauveria bassiana*	*Trichoderma reesei*, *A. oryzae*	90 and 107
Thermolides	HR (PKS-NRPS hybrid)	KS-AT-DH-*C*-MeT-KR-ACP-C-A-T-C_T	*Talaromyces thermophilus*	*A. nidulans*, *E. coli*	108
17 polyketides	NR and HR	Non- reducing and highly reducing	Diverse ascomycete and basidiomycete fungal species	*S. cerevisiae*	109
2-Alkenyltetrahydropyran derivatives including virensol	HR	KS-AT-DH-ER-KR-ACP	*Trichoderma applanatum*	*A. nidulans*, *S. cerevisiae*	66
Valactamide A	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP	*A. terreus*	*A. nidulans*	97
Xylariolide D	HR	KS-AT-DH-*C*-MeT-ER-KR-ACP	*P. crustosum*	*A. nidulans*	110

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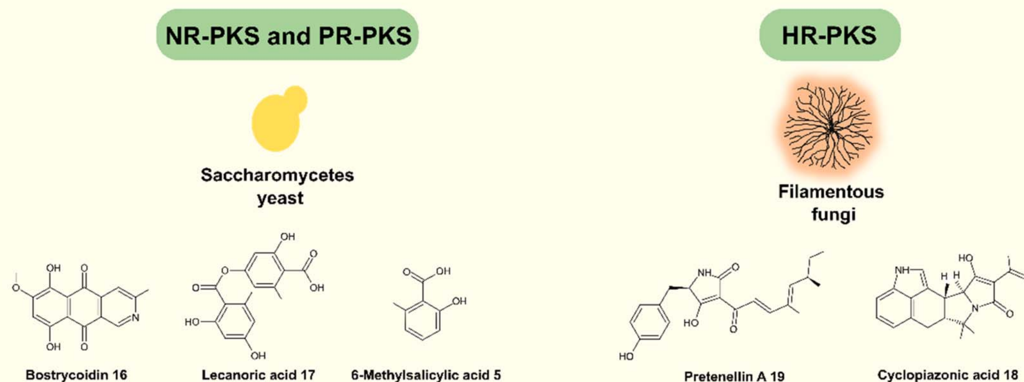

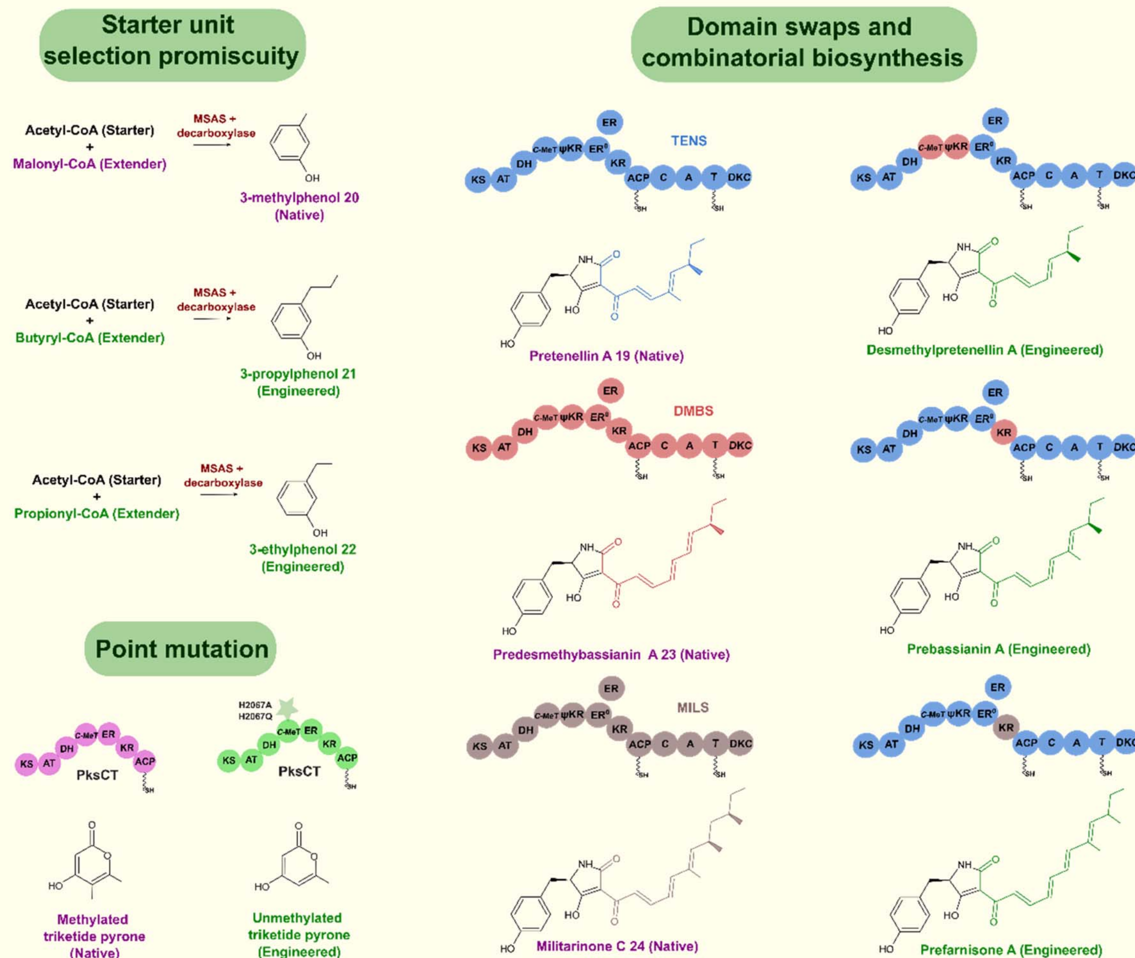

Fig. 4 Design principles for fungal PKS expression and engineering. (A) Heterologous expression of fungal PKSs: non-reducing (NR), partially reducing (PR), highly reducing (HR) PKSs. (B) Engineering of fungal PKSs: 6-methylsalicylic acid synthetase (MSAS), citrinin PKS (PksCT), pretenellin A synthetase (TENS), predesmethylbassianin A synthetase (DMBS), and militarinone C synthetase (MILS). Predominant engineered compounds are shown.

heterologous PKS product could potentially be derivatized due to cross talk with endogenous pathways.[66,71–73]

Although many studies have relied on *S. cerevisiae* and Aspergilli to produce a wide range of fungal PKSs, it is necessary to establish novel heterologous hosts since there is no guaranteed success that all fungal BGCs can be expressed by these hosts. For example, *A. oryzae* and *A. niger* did not yield products from a relatively simple PKS from a lichenized fungus due to unknown reasons.[66] Moreover, product titers for a heterologous PKS may vary across hosts that belong to the same genus. For instance, **5** yields were different when the corresponding PKS, a PR-PKS, was expressed in *A. aculeatus*, *A. nidulans*, *A. niger*, and *A. oryzae*, where *A. niger* was the lowest, even though it is a native producer of **5**.[74] The above examples clearly suggest "one strain fits all products" is not achievable in heterologous production of polyketides. The selection of a heterologous host should depend on the origin and characteristics of a given PKS, such as the taxonomy of the native producer, codon usage, intron processing, PKS type, and the host's native capability to express the selected PKS.

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Recently, non-conventional fungi have been used for the heterologous production of PKSs including *Komagataella phaffii*,[75–77] *Kluyveromyces marxianus*,[78] *Yarrowia lipolytica*,[79–81] and *Saccharomyces boulardii*.[82] Non-conventional yeasts are becoming popular as heterologous hosts because they offer attractive advantages for industrial processes, such as tolerance to environmental stresses, including high temperatures, osmotic pressure, uptake of second-generation feedstocks and recalcitrant substrates, toxins, pH, *etc.*[83,84] Nevertheless, most of the heterologous PKSs that have been expressed in non-conventional yeast are NR-PKS, and it is necessary to challenge them with HR-PKS, as this is the most frequent fungal PKS (Fig. 3). In addition, it is necessary to develop more tools for precise genetic manipulation of non-conventional hosts to allow for precise integration of biosynthetic pathways, as well as to understand the mechanisms of non-conventional yeast stress tolerances.[83,85]

In conclusion, all heterologous hosts offer production advantages and trade-offs. The selection of host will be based on the PKS product, the necessities of the industrial bioprocess, and the final application. Therefore, it is desired to establish novel heterologous hosts and to catalogue existing hosts that we could refer to and select from.

4.2 PKS engineering

Given the multidomain nature of PKSs, there is potential for engineering to expand the diversity of polyketides, which could be used in pharmaceuticals, agriculture and biotechnology-based applications. While the rules for engineering these enzymes are not fully established yet, there have been some notable efforts to guide future engineering efforts.[111] The recent examples of fungal PKS engineering are summarized here based on starter unit selection, point mutation and domain swaps. These approaches represent important steps towards harnessing the full potential of PKSs for the synthesis of novel bioactive compounds with tailored properties and applications.

4.2.1 Starter unit selection promiscuity. A straightforward approach to modify the products of PKSs is to take advantage of the substrate promiscuity of some of their loading domains. For instance, MSAS utilizes acetyl-CoA as a starter unit and malonyl-CoA as an extender unit, to generate **5**, which can then be decarboxylated to form 3-methyl phenol **20** (Fig. 4).[112] However, MSAS and the decarboxylase can produce 3-propylphenol **21** and 3-ethylphenol **22** when heterologously expressed in *S. cerevisiae* by the promiscuous incorporation of butyryl-CoA and propionyl-CoA as starter units (Fig. 4). In addition, PksA, an NR-PKS involved in aflatoxin B biosynthesis in *A. parasiticus* also accepts varied starter units in addition to its native starter substrate hexanoyl, leading to engineered naphthopyrone variants.[113] In native fungal systems, these starter units are often provided by dedicated FASs or HR-PKSs, which generate and transfer the appropriate acyl chains to the NR-PKSs. Studies using alternative starter units as *N*-acetylcysteamine (SNAC) thioesters have shown that structural modifications to the starter unit, particularly at positions distal to the thioester moiety are often tolerated by the PKS and can be fully extended to full chain-length products. These findings illustrate the increased flexibility in the SAT domain of FAS/NR-PKS or HR-PKS/NR-PKS complexes allowing them to accommodate diverse starter units yielding new products.[113]

4.2.2 Functional alterations of PKS domains by mutation. Domain mutations offer a powerful method to dissect and engineer the complex machinery of PKSs. The citrinin PKS (PksCT), an NR-PKS from *Monascus purpureus* was subject to mutation in the *C*-MeT domain.[114] Structural characterization of the PksCT-*C*-MeT domain revealed the presence of N-terminal and C-terminal subdomains placing the domain's active site at their interface. This study also showed that the residue His2067, located in the active site is important for efficient methyl transfer and proposes that *C*-MeT methylation acts as a checkpoint tag that is recognized by the KS domain to determine whether to continue chain extension or release an immature product (Fig. 4).[114] Such an idea implies that to engineer the length of a polyketide product, modifications in the *C*-MeT methylation must be considered. In another study, Cacho *et al.* proposed that the *C*-MeT of LovB, a PKS involved in lovastatin biosynthesis, competes with the KR domain for the substrate. In this case, the domain *C*-MeT was found to exhibit higher catalytic efficiency for the natural tetraketide but showed lower selectivity toward other substrates, prompting the KR domain to act prior to methylation.[115] In a separate investigation, engineering efforts focused on the ER domain from the HR-PKS, directing the biosynthesis of squalestatin.[116] In this case, the point mutations guided by docking simulations were aimed at broadening the ER domain's substrate scope. *In vitro* assays demonstrated successful conversion of non-native substrates with various lengths and methylation patterns by engineered ER domains.[116] These examples not only highlight the utility of point mutations for functional analysis and engineering but also underscore their importance in understanding the evolutionary programming of fungal iPKSs.

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4.2.3 Domain swaps and combinatorial biosynthesis. Domain swap is another method to engineer PKSs, involving the replacement of one functional domain in a PKS with a similar domain from another PKS to reprogram the biosynthetic outcome. Production of 23 unnatural polyketides was achieved by swapping domains of closely related PKSs in an HR-PKS·NR-PKS complex, wherein the HR-PKS synthesizes the starter unit for NR-PKS, leading to the biosynthesis of rare S-type benzenediol macrolactones (BDLs) in *S. cerevisiae*, in which the benzene ring is formed through a C8–C3 aldol condensation.[117] Using five closely related HR-PKS·NR-PKS complexes as domain donors, combinatorial biosynthesis was performed with various HR-PKS·NR-PKS combinations, SAT domains, and TE domains.[117] In a subsequent exploration, 10 additional unnatural BDLs were produced through alternative HR-PKS·NR-PKS complex combinations. This study suggests that the SAT domain of the NR-PKS competes with further chain extension and reduction by the KS and KR domains of the HR-PKS. As a result, acyl intermediates of varying chain lengths and oxidation states are transferred and subsequently processed differently by downstream biosynthetic enzymes.[118] Another investigation examined *in vitro C*-MeT domain swaps between six different PKSs belonging to type I NR-PKSs. When paired with non-cognate minimal PKSs, these domain swaps led to methylated products, indicating that methylation programming is intrinsic to the *C*-MeT domain.[119]

Further research focused on the closely related HR-PKS-NRPS (non-ribosomal peptide synthetase) systems of pretenellin A **19** synthetase (TENS), predesmethylbassianin A **23** synthetase (DMBS), and militarinone C **24** synthetase (MILS), whose products display variations in chain lengths and methylation patterns.[3] TENS served as the base system for homologous swaps of *C*-MeT, ψKR, and KR domains from DMBS and MILS.[3] Through this engineering approach, products with diverse methylation patterns and chain lengths were generated (Fig. 4). The findings suggest that methylation programming may be influenced by competition between *C*-MeT and KR domains, while chain length selectivity could stem from competition between KR and the off-loading NRPS.[3] In a follow-up study by the same research group, exchanging the homologous substrate-binding helix adjacent to the KR active site resulted in chain length variation.[52]

5. Perspectives and guidance for future engineering efforts

Two key perspectives that may be relevant for those interested in engineering fungal PKSs can be outlined from this highlight article: (i) the selection of a suitable expression host and (ii) the design of PKS engineering.

5.1 Host selection for heterologous production of PKSs

We propose that heterologous expression efforts prioritize more PKS-proficient species while deemphasizing Saccharomycetes yeasts. Although these yeasts are highly tractable, they require extensive modifications to produce polyketides, which limit their product scope and makes them less versatile compared to other expression hosts. Instead, new heterologous hosts should be selected based on a combination of advantageous traits such as wide native PKS product scope, metabolic proficiency, genetic tractability, and the ability to utilize sustainable substrates.

For instance, unicellular species of the genus *Exophiala* from the Eurotiomycetes class,[120] known as black yeasts, exhibit several desirable traits such as a wide native PKS product scope. These species are tractable, have relatively low metabolic backgrounds, require shorter engineering cycles compared to filamentous fungi, and exhibit native metabolism dedicated to the copious production of polyketides.[121] However, they are not widely adopted, and the availability of genetic engineering tools for them remains limited.

It could be argued that, due to its long history as a laboratory model, extensive host engineering could eventually make *S. cerevisiae* a viable PKS expression host. However, with the increasing accessibility of low-cost genomics and genome editing technologies, genetic engineering tools for alternative fungal hosts could be rapidly expanded, allowing for the adoption of more naturally proficient polyketide producers.

5.2 Design of PKS modifications

Current PKS engineering approaches mainly rely on rationally selected mutations and domain swaps. Domain swaps are an efficient engineering method when using parts from closely related PKSs, suggesting that evolutionary constraints operate among PKS domains. These constraints can be addressed using phylogenomic methods to guide the selection of parts for chimeric PKSs. However, conventional domain swaps have inherent limitations, as they merely reshuffle the chemical space that nature has already created. To truly expand product diversity beyond the natural repertoire, new approaches to PKS engineering are needed.

One promising avenue is the use of generative protein artificial intelligence (AI) models. For instance, ESM3 has demonstrated remarkable potential in protein engineering by creating green fluorescent proteins-like proteins from scratch.[122] It is reasonable to envision that AI models could be fine-tuned to generate PKSs. To generate functional PKSs, such a model would need to learn the co-evolutionary dependencies between catalytic domains and be capable of capturing how subtle sequence variations drive changes in programming logic. By learning from known evolutionary trajectories, a generative AI model could generate PKS sequences that are not only novel but also evolutionarily plausible, thereby increasing the likelihood of producing functional enzymes.

Finally, we hope that by highlighting both the successes and the understudied aspects of PKS engineering, this highlight article encourages other natural product scientists to join the growing field of PKS engineering, whether by leveraging well-established methods to create new molecules or by addressing key knowledge gaps, such as fungal PKS evolution and programming.

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6. Conflicts of interest

JDK has financial interests in Amyris, Ansa Biotechnologies, Apertor Pharma, Berkeley Yeast, Cyklos Materials, Demetrix, Lygos, Napigen, ResVita Bio, and Zero Acre Farms. The other authors declare no competing interests.

7. Data availability

The genome sequences used for this article are all available at the gene bank and their accession numbers are indicated in supplementary information (SI) file 1. The genomes were annotated using the scripts available at: https://github.com/WeMakeMolecules/fun-git. The PKS and their domain organization were extracted using scripts available at: https://github.com/WeMakeMolecules/Megasynthase_string_miner.

Supplementary information: file 1 includes values for PKSs frequency by type and taxonomy as well as a complete list of the PKSs used to obtain Fig. 3 is available. See DOI: https://doi.org/10.1039/d5np00020c.

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