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Technical advances to accelerate modular type I polyketide synthase engineering towards a retro-biosynthetic platform

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Abstract

Modular type I polyketide synthases (PKSs) are multifunctional proteins that are comprised of individual domains organized into modules. These modules act together to assemble complex polyketides from acyl-CoA substrates in a linear fashion. This assembly-line enzymology makes engineered PKSs a potential retro-biosynthetic platform to produce fuels, commodity chemicals, speciality chemicals, and pharmaceuticals in various host microorganisms, including bacteria and fungi. However, the realization of this potential is restricted by practical difficulties in strain engineering, protein overexpression, and titer/yield optimization. These challenges are becoming more possible to overcome due to technical advances in PKS design, engineered heterologous hosts, DNA synthesis and assembly, PKS heterologous expression, and analytical methodology. In this review, we highlight these technical advances in PKS engineering and provide practical considerations thereof.

Keywords

polyketide synthase, retro-biosynthetic analysis, protein engineering, heterologous expression, biofuels, bioproducts

1. Introduction

Retrobiosynthesis, an approach to design *de novo* biosynthetic pathways, has enabled the production of various useful chemicals, including biofuels [1] and drugs [2]. In this approach, design starts from the target molecule and proceeds backwards to precursors by considering stepwise biochemical reactions. Modular type I polyketide synthases (PKSs) have the potential to be a versatile retro-biosynthetic platform for its collinear and modular biosynthetic logic. Native PKSs catalyze the formation of carbon-carbon bonds in an assembly-line manner to synthesize the scaffolds of complex natural products, such as macrolides (*e.g.* 6-deoxyerythronolide B) [3], polyenes (*e.g.* α-lipomycin) [4], and polyethers (*e.g.* salinomycin) [5]. In this biosynthetic process, each module of a PKS catalyzes a two-carbon elongation followed by potential reductions. During elongation, the acyltransferase (AT) domain loads a specific malonyl-CoA analog onto the phosphopantetheinyl group of the acyl-carrier protein (ACP) domain. This phosphopantetheinyl group is derived from the essential post modification of PKS via phosphopantetheinyl transferases (PPtases). After loading, the ketosynthase (KS) domain, which is primed with an acyl group (a starter acyl group or an acyl chain formed in the previous module), catalyzes a Claisen condensation to fuse the acyl group with the decarboxylated malonyl unit. After elongation, if present, the ketoreductase (KR) domain stereoselectivity reduces the β-ketone to a hydroxyl group. The dehydratase (DH) domain can then catalyze a dehydration, resulting in the loss of this hydroxyl group to form an α-β double bond. Finally, the enoylreductase (ER) domain can reduce this α-β double bond to form a saturated bond. The oxidation state of the β-carbon depends on the presence of reductive domains in each module. Following rounds of elongation and reduction, the acyl-chain is released by a thioesterase (TE) domain by cyclization to form a macrolactone or hydrolysis to form a linear product. The collinearity of the PKS biosynthetic process results in a diversity of products due to different AT substrate specificities and varying degrees of reductions in each module, and the different module organizations (**Fig. 1**).

Many fundamental studies used engineered PKSs as a retro-biosynthetic platform by conducting domain modifications, domain swaps, and module rearrangements; these results have been

extensively reviewed elsewhere [6–9]. These studies indicated that there is still a large gap between the promise of PKS engineering and reality, especially for constructing novel multi-module PKSs. Hence, compared to complex natural products, commodity chemicals or biofuels bearing simple structures are more realistic targets for PKS engineering [8]. The production of these targets only requires connecting a limited number of PKS modules (usually ≤ 2). However, even for these simple targets, unique challenges emerge when engineering PKSs to produce compounds *in vivo*. Besides the fundamentally mechanistic understanding of PKS biosynthesis, these challenges stem from the practical difficulties in novel PKS design, host selection, large DNA fragment synthesis and assembly, target production, and product detection (**Fig. 1**). In this review, we highlight the state-of-the-art technical advances that deal with these challenges and provide practical considerations for PKS engineering.

2. PKS design

To determine a target molecule for production using PKSs, both economic and technical considerations must be evaluated. First, the candidate should be assessed for cost and energy efficiency. Next, retro-biosynthetic analysis can be used to evaluate the feasibility for the candidate molecule to be made through PKS mechanisms. Once a candidate is established to be a feasible target molecule, an initial biosynthetic pathway may be designed using software tools (**Fig. 2**).

Deciding on a target molecule depends on economic feasibility, which reduces to evaluating the value of the candidate against the cost of its production. An estimate of the candidate's monetary value can be established based on existing commercial value, if known. Otherwise, the value should be estimated according to the candidate’s potential applications or molecules with similar functions [10]. To determine production cost, a techno-economic analysis that considers molecular precursors, energy requirements, and production time may be performed [11]. Finally, it is important to consider if the target molecule can be produced in a more practical or economically favorable means through other biosynthetic pathways. While PKSs have recently been engineered to produce short-chain ketones, potential gasoline blending agents, from plant biomass at titers of 1 g/L [12], fatty acid and isoprenoid

synthesis are currently more established production pathways with higher yields [13]. This holistic economic analysis of the target molecule can guide the extent of engineering efforts required for production.

Once established as economically feasible, a retro-biosynthetic analysis can determine a possible pathway for attaining the compound. In this process, the candidate should be deconstructed by carbon-carbon bond breaking according to PKS biosynthetic logic. Engineered PKSs modular nature can be leveraged to programmatically produce non-natural compounds. As such, extensive research has been performed to understand the rules concerning domain swapping to control chain extender unit selection at the acyltransferase (AT) domain and the degree of β-carbonyl reduction by the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) [14–16]. For example, the swapping of non-native ATs has been successfully achieved to switch from methyl-malonyl to malonyl extension [17], and research has been conducted to discover AT's that accept extender units with exotic sidechains (phenyl, allyl, etc.) [18, 19]. The principles of domain swapping in PKSs are maturing, and there is a wealth of information to determine viability for novel PKS products [9, 20]. In parallel, considerable progress has been made to engineer PKS domains with more traditional protein engineering methods (*e.g.* mutagenesis) [21]. With the expanding research on the engineering possibilities of PKSs, the range of molecules that are accessible through PKS biosynthetic pathways is continuously growing.

With a viable target molecule determined, software enables the rapid identification of natural PKS domain candidates to be combined into a suitable catalytic system. Most recently, the free, online software package ClusterCAD [22] has been developed for *in silico* engineering of PKS pathways to achieve synthesis of a specified product. ClusterCAD semi-quantitatively determines the similarity of the target molecule to the native substrates of an annotated database of biosynthetic gene clusters. Users may additionally specify design constraints, such as native substrate size similarity or AT extender selectivity, to narrow the number of possible routes to the product. The software then proposes biosynthetic pathways that combine components from multiple PKSs that meet the design criteria. ClusterCAD is also just one of the many available in silico tools that help guide the engineering process.

120 The identification of biosynthetic loci for every known secondary metabolite class [23], the structure-
121 based sequence analysis of PKSs [24], and a crowd-sourced database of microbial PKS and NRPS gene
122 clusters [25] are all accessible from present software tools. Despite such advances in our understanding
123 of PKS engineering, not all design rules for these enzymatic systems have yet been elucidated. As a
124 result, an experimental, combinatorial approach to produce the output PKS systems is often still the
125 most effective method for successful PKS engineering.

3. Host selection

The growth properties of the host organism are important to take into account when selecting a heterologous host for PKS production. Many natural polyketide producing microbes are not culturable in laboratory conditions [26] or grow very slowly in laboratory conditions as is the case with lichen [27] and thus are not suitable hosts for heterologous expression. Bacteria from the genus *Streptomyces* are common heterologous hosts for PKSs due to their status as prominent PKS producers but they can frequently present issues for industrial scale-up including slow growth and the tendency to form mycelial clumps [28], the latter of which has been shown in some cases to be important in the activation of biosynthetic gene clusters [29]. Despite that, *Streptomyces* continue to be one of the most popular industrial heterologous hosts for polyketide products and major efforts have been put in place to engineer strains that do not form clumps and grow quickly [30]. Another growth property of interest includes the carbon and energy sources used by the heterologous host. Heterologous hosts such as *Pseudomonas putida* which consume a wide variety of lignin compounds as a carbon source [31] or the cyanobacterium *Synechococcus elongatus*, which can use light as an energy source and CO_2 as a carbon source [32] have been adapted for the production of PKSs (**Table 1**).

The availability of genetic tools for a particular host is of great aid to the task of genetically engineering a host to heterologously produce polyketides. Most common heterologous hosts have well developed methods for transformation, conjugation, or transduction of vectors. Of particular importance for controlling expression levels heterologously is a comprehensive list of compatible promoters and RBSs (**Fig. 3(A)**). These are well known for classic heterologous hosts like *E. coli* [33] and there have been successful efforts to develop promoter-RBS libraries for *Streptomyces* [34]. Ultimately, genomic integration of engineered gene clusters is desired in industrial heterologous host strains as it eliminates the need for selection pressures such as antibiotics and provides a more stable and consistent expression of genes [35]. Tools for genomic integration─such as site-specific integrases, homologous recombination-based integration vectors, and CRISPR-Cas9─are widely available for common hosts like *E. coli* and have also been adapted for *Streptomyces* [36–38]. As these tools continue to be

developed for *Streptomyces* and other suitable PKS hosts the ability to heterologously produce PKSs will continue to improve.

When selecting a host for heterologous expression one must consider the existing metabolic infrastructure to ensure that the host contains all the precursors and tailoring enzymes needed to produce the desired polyketide. Among the most important things to consider is the expression of a compatible PPTase [39] (**Fig. 3(B)**). As mentioned above, PPTases are responsible for activating ACPs by modifying the active site serine with a phosphopantetheinyl moiety. To ensure the heterologous host can phosphopantetheinylate the engineered PKS in question it is important to ensure the host expresses an appropriate PPTase. While many natural type I PKS producers such as *Streptomyces* have native PPTases, many common hosts do not and a PPTase must be heterologously expressed. One of the most common PPTases used in heterologous PKS expression is Sfp from *Bacillus subtilis* because it is capable of phosphopatetheinylating a wide variety of type I PKS and NRPS carrier proteins [40, 41]. However, Sfp may not be active on certain PKSs [42], thus it is important to check for phosphopantetheinylation via proteomics methods [43]. Another important prerequisite is the availability of precursors such as extender units and starter acyl-CoAs. The most common extender units used by PKSs are malonyl-CoA and methylmalonyl-CoA [44] although there do exist a variety of rare extender units as seen in the biosynthesis of zwittermycin [45], chlorizidine [46], or unnatural extender units [47] that can be incorporated by engineered PKSs (**Fig. 3(C)**). While all organisms produce malonyl-CoA for fatty acid synthesis the ability to produce methylmalonyl-CoA is absent from many common hosts such as *E. coli* and *S. cereviseae*. This challenge was overcome in *E. coli* by the development of the K207-3 strain which has both Sfp and a propionyl-CoA carboxylase present to produce intracellular methylmalonyl-CoA [48]. Ultimately a researcher must determine what precursors and tailoring enzymes are necessary for the production of their desired molecule and must take steps to either engineer the heterologous host to produce these precursors or find a heterologous host that naturally produces these precursors and tailoring enzymes.

The stability and toxicity of the target molecule in the heterologous host must be evaluated. It is possible for the target molecule to be toxic to the host, therefore it is important to do a growth inhibition assay if a sample of the target molecule is available. Additionally, it is possible for the target molecule to be consumed as a carbon source by the host organism. This can be determined by growing the organism on the target molecule as a sole carbon source if possible. Finally, it is possible for the target molecule to be modified or unstable in the cell and this can be detected by incubating the target molecule in cell lysate before extraction and analysis.

4. DNA synthesis and assembly

After the target PKS organization and host are determined during the design stage, the careful consideration of the strategy for DNA synthesis and assembly of PKS genes are needed in the build stage (**Fig. 4**). To expedite the whole engineering process, automation of the steps in the build stage is preferable, although some challenges exist.

PKSs are typically encoded by a series of genes clustered in the genome of the producing organisms [49]. Thus, the cloning and expression of PKSs can be achieved by acquiring the genomic DNA (gDNA) harboring the entire cluster from a gDNA library construction. In addition to the phage-mediated homologous recombination-based methods [50], transformation-associated recombination (TAR)-based techniques have been developed to capture large biosynthetic gene clusters from environmental DNA or gDNA samples. These techniques utilize the native *in vivo* homologous recombination of budding yeast *Saccharomyces cerevisiae*, which occurs in much higher frequency compared to ligation or non-homologous end joining methods [51]. On the other hand, *in vitro* homology-based methods such as Gibson assembly and sequence- and ligation-independent cloning (SLIC) methods are advantageous over *in vivo* methods in the turnaround time, although the construct size is typically smaller.

If the PKS genes are heterologously expressed and require DNA refactoring like codon optimization, it is most likely that *de novo* DNA synthesis is needed. Although the trends of price per base for DNA sequencing and column-based oligonucleotide synthesis seem to have reached plateaus in recent years, the cost for gene synthesis continues to decrease [52]. As an alternative to outsourcing DNA synthesis, array-based gene synthesis may be carried in house, followed by error correction and verification [53]. In addition, enzymatic *de novo* synthesis of oligonucleotides with terminal deoxynucleotidyl transferase (TdT)-deoxynucleotide triphosphates (dNTPs) conjugates may serve as a promising basis for enzymatic oligonucleotide synthesizer [54]. Online tools such as JBEI ICE public registry [55] and SynBioHub [56] may facilitate users to search and share the designs of DNA parts.

Because PKS genes are typically modular, the modules are usually exchanged during PKS engineering in combination with various promoters, terminators, ribosome-binding sites (RBSs), linker domains, and other parts. If DNA cloning is high-fidelity, sequencing the individual parts may be avoided to reduce cost and effort and save time. Different parts could then be assembled by several methods including yeast assembly [57], Gibson assembly [58], and Golden Gate assembly [59]. In the case of PKS gene construction, although yeast assembly takes a few days, it has the advantages of generating large final gene constructs and promoting recombinational joining of unrelated DNA fragments with the aid of "stitching oligonucleotides", compared with *in vitro* methods [60]. The construction of shuttle vectors that allow amplification, replication, and expression in *S. cerevisiae*, *E. coli* and the selected host would greatly facilitate this process, as well as the development of automation method such as high-throughput transformation of *S. cerevisiae* using liquid handling robots [61].

5. Production and analysis

The introduction of the assembled PKS genes into the selected host yields the candidate strain, which needs the appropriate conditions to produce the target molecules. After production, analytical methods allow researchers to assess the production of the desired product and/or possible shunt products

to evaluate the performance of the engineered PKS. Finally, troubleshooting and optimization can be facilitated by detection of intermediates and high throughput screens.

A major consideration in the production stage is the selection of a growth medium. A well-selected medium facilitates the strain's growth and PKS biosynthesis [62, 63] and ultimately increases the chance of detecting the final product [64], considering most engineered PKSs result in a decreased production [6, 8]. The media preferences for the PKS production vary from host to host. For common heterologous hosts the media selection can be straightforward since the media for these organisms are widely used and commercially available. Additionally, PKS production in these organisms is clear, as the precursor biosynthetic genes and PPtase gene are usually under the control of strong constitutive promoters [48, 65, 66]. Hence, rich media are usually the first choice for these hosts. Examples include LB medium used to produce lactones [67, 68] and short chain ketones [17] by engineered PKSs in *E. coli*, while YPD medium was used to produce lactones by engineered PKSs in *Saccharomyces cerevisiae* [65]. However, as most native PKS producers are not model organisms, the media selection could be challenging when these organisms are chosen as hosts. Rich media usually suitable for cell growth may not be suitable for PKS production in these hosts, since these hosts typically produce their native PKSs under nutritional deficiencies [69]. Therefore, for the PKS production in these non-model hosts, production mediums screens are usually required to produce the desired molecules [64, 70] (**Table 1**).

Once production of the target molecule is confirmed, media optimization is required to maximize the yield and reduce cost. This optimization is crucial for low-value commodity chemicals/biofuels production. In this process, carbon source, nitrogen source, phosphate, and other nutrients are carefully adjusted via a one-factor-at-a-time method or statistical method [71]. In the case of short chain ketone production by an engineered PKS in *S. albus*, when the carbon source was supplemented by the plant-biomass hydrolysates the product titer increased more than four-fold and further titer increases were achieved by feeding specific acyl-CoA precursors [12]. Aside from

traditional methods, recently developed machine learning algorithms have great potential to accelerate the media optimization process [72].

Researchers receive feedback regarding the functionality of their engineered PKS when analytical methods are used to detect reaction products. Gas/liquid chromatography coupled to mass spectrometry (GC/LC-MS)-based methods are usually the first choice for analysis [12, 73, 74] because of their low detection limit for commodity chemicals and biofuels. However, these methods only detect released molecules and leave the PKS biosynthetic pathway a black box. Engineered PKSs frequently fail to produce any free products *in vivo* in the first Design-Build-Test-Learn (DBTL) cycle. Thus, other analytic approaches are needed to debug these failed PKSs for the next DBTL cycle. Transcriptome [75] and proteomics [76] analysis are well developed to detect the expression of PKSs. Ppant ejection methods are designed to detect on-line PKS intermediates [43]; this method was used to optimize an adipic acid producing PKS *in vitro* [74] (**Fig. 5(B)**). Together, these analytical methods can be used effectively to detect the production of target molecules or help troubleshoot malfunctioning modules.

Aside from these 'rational' approaches, high throughput screening/selection is another possible route to create a functional PKS *in vivo*. With well-developed mutagenesis methods, generating a big mutant library is feasible for a specific PKS [77]. The methods used to select or screen for an improved mutant result in the largest bottleneck in a high throughput approach. Screening via GC/LC-MS systems is a possibility [78], however, despite the availability of high throughput GC/LC-MS systems that have reached the speed of several seconds per sample [79], it is still ineffective when the size of mutagenesis library becomes large ($>10^{10}$). High throughput detection could be achieved by tying the production of the target molecule with a detectable signal or with the viability of the hosts [80]. For most compounds lacking bioactivity or a chromophore, biosensors are usually required to perform the connections. Although to our knowledge there are no reported cases of a biosensor being coupled with type I PKS production *in vivo*, some biosensors are reported to detect chemicals that are made or can be possibly made by engineered PKSs, such as diacids [74, 81], lactams [82–84], and pyrones [85, 86]. These compounds are promising targets for high throughput PKS engineering (**Fig. 5(B)**).

6. Conclusions

Currently, engineered PKSs can produce several products that are not naturally occurring products in microorganisms. These accomplishments benefit from an expanded understanding of type I modular PKS biosynthetic mechanisms, as well as technical advances summarized here. Similar advances will continue to emerge and promote PKS engineering to be a reliable retro-synthetic platform for the production of commodity chemicals and biofuels. We speculate that over the next few decades, an automatic solution to produce new chemicals by engineered PKS will be available. This technology will include an automatic retro-synthesis software, super hosts for PKS production, automatic DNA synthesis and assembly platforms, and high-throughput production and analytical methods. This has begun to be realized since these technologies have been developed in related fields such as PKS derived natural products discovery [80, 87–89]. By harnessing these advances in PKS engineering and synthetic biology technologies, we will be able to engineer PKS to access an extensive chemical space.

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Conflict of interest

J.D.K. has financial interests in Amyris, Lygos, Constructive Biology, Demetrix, Napigen, Maple Bio, and Apertor Labs.

311 **Table 1.** Selected cases of engineered PKSs used to produce target chemicals in microbial hosts.

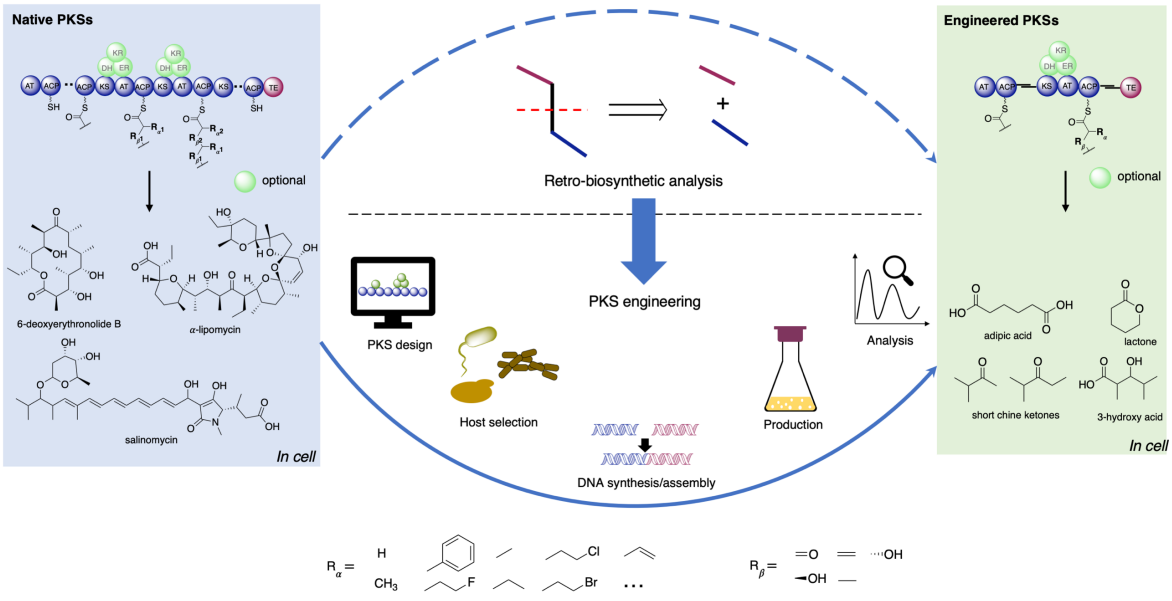
312

Organism	Strain	Media	Product	Highest titer	Reference
Escherichia coli	K207-3	Terrific Broth (TB)	Short-chain ketone	4 mg/L	[17]
	K207-3	Luria-Bertani (LB)	Triketide lactone	23 mg/L	[68]
	BAP1	LB	6-deoxyerythronolide B (6-dEB) analog	1 mg/L	[90]
	K207-3	LB	Long side chain triketide lactone	14.6 mg/L	[91]
Synechococcus elongatus	AMC2302	BG11	Multimethyl-branched fatty acid	N/A[a]	[32]
Streptomyces albus	J1074	Modified 042	Short-chain ketone	1 g/L	[12]
Streptomyces coelicolor	OP	SCFM6-2	Triketide lactone	500 mg/L[b]	[92]
	CH999	R2YE	Triketide lactone	3 mg/L	[93]
Streptomyces lividans	K4.114/ K4.115	R5 or R6	6-deoxyerythronolide B (6-dEB) analog	20 mg/L	[94, 95]

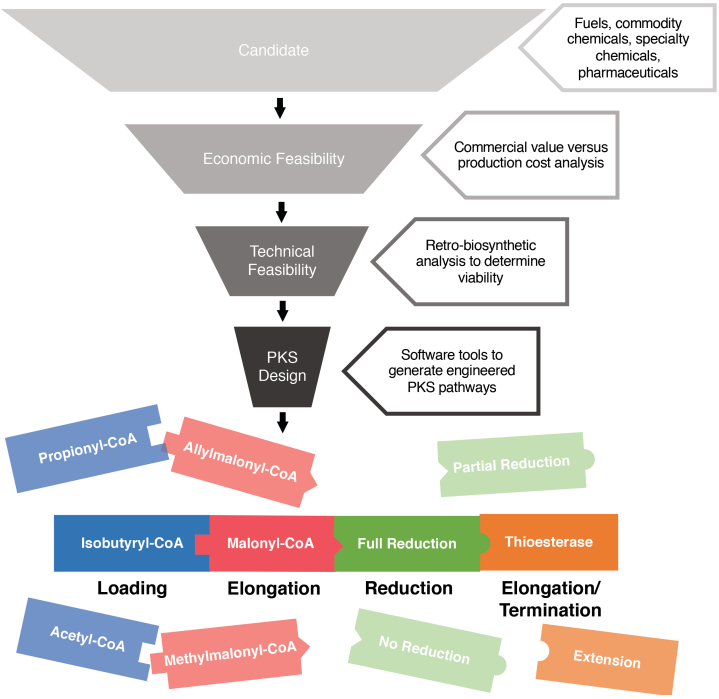
Streptomyces venezuelae	ATCC 10712	042	3-Hydroxycarboxylic acid	13.8 mg/L	[64]
	N/A[a]	SCM	Triketide lactone	N/A[a]	[96]
Saccharomyces cerevisiae	BJ5464	YPD	Triketide lactone	1 mg/L	[65]

[a] not available
[b] with diketide feeding

Fig. 1. Retro-biosynthetic analysis is a potential platform to bridge native PKSs and engineered PKSs, and the realization of this potential depends on the technical advances in PKS design, host selection, DNA synthesis and assembly, target production, and product analysis. The logic of PKS biosynthesis is illustrated in native PKSs. Acyltransferase (AT) loads the malonyl-CoA analogs (different R_α) to the acyl carrier protein (ACP). Catalyzed by ketosynthase (KR), the loaded malonyl-ACP decarboxylates and condenses with upstream acyl-ACP intermediate for chain elongation. Optional reduction domains (ketoreductase (KR), dehydratase (DH), and enoylreductase (ER)) reduce the β-ketone to an alcohol, double bond, or saturated bond (R_β). After rounds of elongation and reductions, the final acyl chain is released by a thioesterase (TE) to form a macrolactone or linear product. Elongation domains are represented as blue sphere, optional reduction domains as green spheres, and release domains as purple spheres.



328 **Fig. 2.** Economic and technical considerations in PKS design



329

Fig. 3. Heterologous host selection must take into account the genetic tools available for the host as well as the metabolic precursors produced by the host. (A) Promoter/RBS libraries help control transcription and translation levels of heterologous proteins. (B) Appropriate PPTases are needed to adequately phosphopantetheinylate heterologous ACP domains. (C) Malonyl-CoA and methylmalonyl-CoA serve as the most common extender unit precursors for type I PKSs though rare extender units exist. RBS - ribosomal binding site, PPTase - phosphopantetheinyl transferase, CoA-SH - coenzyme A, 3',5'-PAP – 3',5'-phosphoadenosine phosphate.

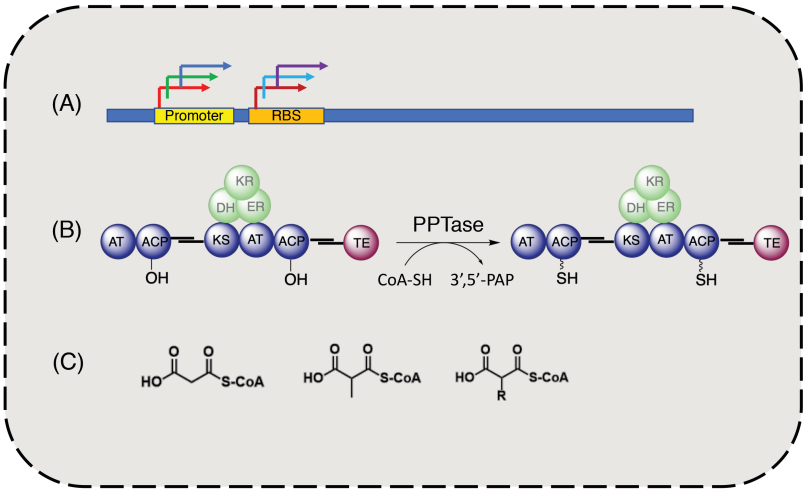


Fig. 4. DNA synthesis and assembly in new PKS construction. Purple square represents corresponding homology region for yeast assembly and Gibson assembly, or corresponding sticky end for Golden Gate assembly. gDNA - genomic DNA, RBS - ribosome binding site, Loading/Extension/Reduction/Termination - different modules of PKS genes, Linker - linker domains.

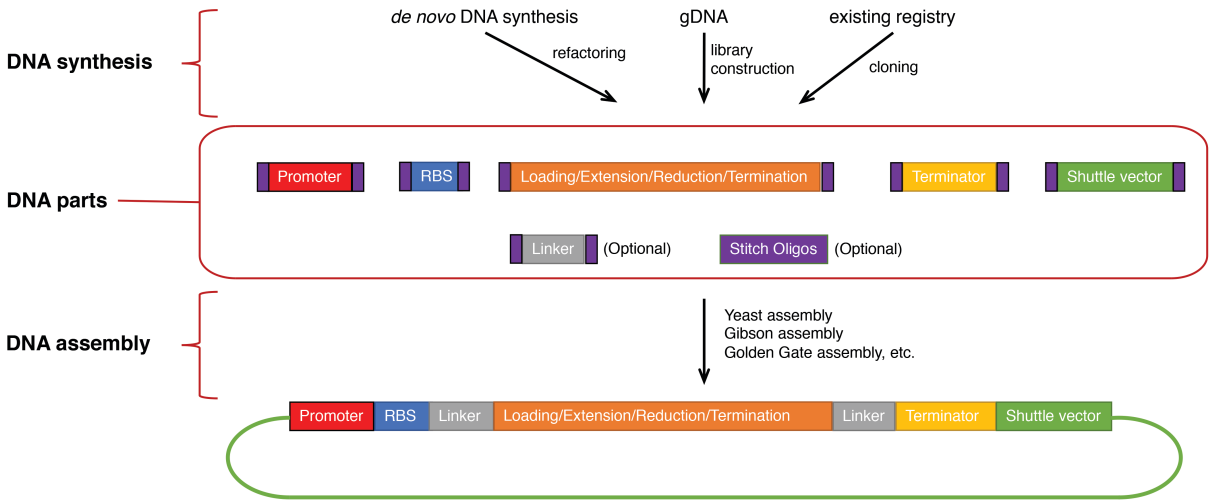
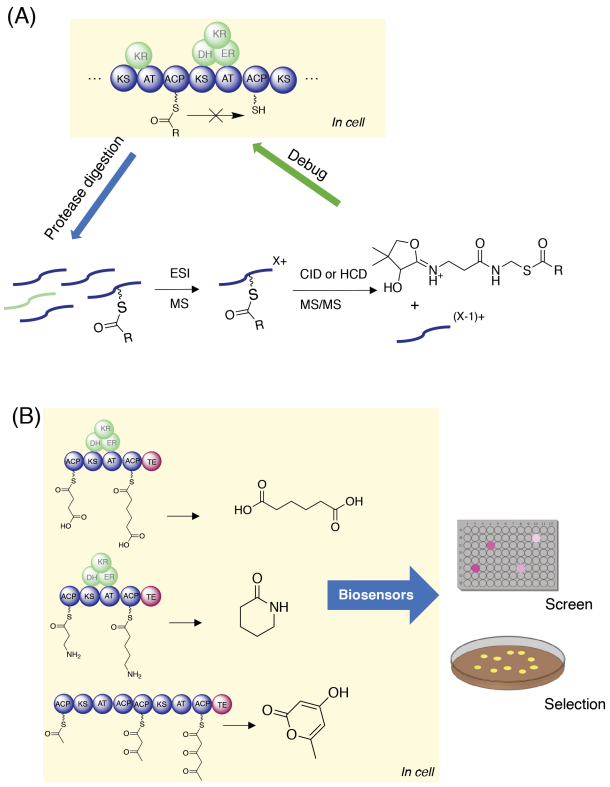


Fig. 5. Proposed methods to troubleshoot the stalled PKS in the host: A) Ppant ejection, B) high throughput screen/selection. ESI - electrospray ionization, MS - mass spectrometry, CID - collision-induced dissociation, HCD - higher-energy collisional dissociation, MS/MS - tandem mass spectrometry.



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