Advances in Metabolic Engineering of L-Pipecolic Acid Supply in Tacrolimus Biosynthesis

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ABSTRACT

Tacrolimus (FK506), the first-line immunosuppressant for organ transplantation, is produced by fermentation of Streptomyces tsukubaensis. L-pipecolic acid constitutes the piperidine ring moiety of FK506; its synthase FkbL exhibits intrinsically low catalytic efficiency, representing the most flux-constrained step in the entire biosynthetic pathway. This review systematically compares recent strategies to overcome this bottleneck-including precursor pathway enhancement, competitive branch redirection, transcriptional and translational regulatory engineering, and fermentation optimization-with a particular focus on structure-guided enzyme design, multi-omics-driven translational optimization, and multi-target synergistic engineering. The current research shows that the hierarchical transcription control efficiency combining multi-copy fkbn and multi-gene cassettes is the best. "Structural Guide" engineering design and rare codon optimization are also new methods to increase production. The future research directions are FkbL directed evolution, the replacement of the whole rare codon, the discovery of indirect control factors, and the topic of simulated biosynthesis using CRISPR.

Keywords: Tacrolimus, FK506, L-Pipecolic Acid, Fkbl, Metabolic Engineering, Streptomyces Tsukubaensis.

1. INTRODUCTION

FK506 was first discovered from Streptomyces tsukubaensis No. 9993 in 1987 [1]. Owing to its immunosuppressive potency 10-100 times greater than cyclosporine, it rapidly became the gold standard for post-transplant management and and its clinical use was supported by subsequent transplantation studies [2]. Current global annual sales exceed USD 3 billion [3]. However, wild-type fermentation titers remain merely 20-50 mg/L, insufficient for industrial-scale production.

The FK506 backbone requires coordinated action of over 30 enzymatic proteins [3]. Among these, FkbL-catalyzed conversion of L-lysine to L-pipecolic acid has been identified as the most critically constrained node owing to its low catalytic turnover rate. This FkbL homologous protein/Rapl-like enzyme absolutely plays a necessary role in making pipecolate, and its properties were first known through the structure of rapamycin [4]. L-pipecolic acid is not only an important source of piperidine ring, but also plays an important role in closing macrolactone ring. In the view of investigating metabolic trend, the value of strong adjustment trend in FkbL stage is higher, and the feedback inhibition of Ask/LysC on lysine route is the second adjustment point. Just putting your hand on one point and forgetting the other is like opening the upstream valve with an interceptor flowing in series and keeping the downstream interception unchanged, which requires multi-target coordination.

Barreiro et al. recently put forward an overall review on tacrolimus [3]. In contrast, the review only focuses on the places where L-pipecolic acid is in short supply, and investigates the characteristics of biosynthetic pathways, the changes of metabolic flow direction, the design of regulation mechanism of transcription and translation, the adjustment of fermentation, and the recent recent breakthroughs in multi-omics analysis [5], structure-guided enzyme design [6], and rare codon translational optimization [5]. In addition, we know that if the redundant polyketide roads are merged with the general acyl, the production of FK506 will be reduced, and if these fusion roads are blocked, the metabolism will flow to the target product [15].

2. THE CENTRAL ROLE OF L-PIPECOLIC ACID IN THE FK506 ASSEMBLY LINE

2.1 Overview of FK506 Biosynthesis and the Rate-Limiting Role of FkbL

The biosynthesis of FK506 proceeds through four stages [3]. In the first stage, chorismic acid is converted by FkbO to generate DHCHC (4,5-dihydroxycyclohex-1-enecarboxylic acid), which serves as the starter unit [7]. In the second stage,

the type I polyketide synthase (PKS) system, comprising FkbA, FkbB, and FkbC, sequentially catalyzes the condensation of two malonyl-CoA, five methylmalonyl-CoA, two methoxymalonyl-ACP, and one allylmalonyl-CoA units over eleven rounds of chain extension [8]. In the third stage, FkbP couples L-pipecolic acid to the polyketide chain via an amide bond and simultaneously promotes cyclization to form the 23-membered macrolactone ring. In the final stage, FkbM and FkbD catalyze C31 O-methylation and C9 oxidation, respectively, conferring full biological activity on the molecule [9].

From a metabolic flux perspective, DHCHC and the extender units can be replenished by enhancing primary metabolism, whereas L-pipecolic acid is exclusively dependent on FkbL with no parallel pathway available for compensation. This "single-point dependency" means that any restriction at FkbL immediately leads to insufficient precursor supply for the entire assembly line.

2.2 Catalytic Properties of FkbL and Structural Breakthroughs

FkbL, a homologous protein of the RapL lysine cyclodeaminase characterized in the rapamycin pathway, catalyzes a three-step cascade reaction-oxidative deamination, intramolecular cyclization, and imine reduction-dependent on NAD^+ as a cofactor [4]. In vitro enzymatic characterization has revealed that its Km for L-lysine is in the millimolar range and its kcat is markedly low [4]. Schulz et al. [6] recently achieved a breakthrough by solving the crystal structures of the FkbL homolog PipAf from Actinoplanes friuliensis in complex with NADH (at 1.3 Å resolution) and with its substrate lysine (at 1.4 Å resolution). Based on this structural information, the authors replaced isoleucine 91 of PipAf with valine (Ile91Val); this rationally designed mutant increased FK506 yield by approximately 100% compared with the wild type in S. tsukubaensis. This work provides the first experimental demonstration that rational design guided by enzyme structural analysis can effectively overcome the intrinsic catalytic efficiency bottleneck of the FkbL enzyme family.

2.3 Metabolic Network Constraints on Lysine Precursor Supply

Lysine biosynthesis is located in the last branch of amino acid metabolism network of aspartate family. The first important stage is the catalysis of aspartate kinase (Ask/LysC), but it is strongly inhibited by the final product lysine. dihydrodipicolinate synthase (DAPA) is also a branch of lysine pathway and threonine/methionine pathway, which determines the distribution of metabolic flow [10]. This dual regulation mechanism strongly limits the flow of substrates available for FkbL. Therefore, any engineering strategy must also support increasing the pool of upstream lysine [11].

3. METABOLIC FLUX REDIRECTION

Wang et al. proved that large-scale expression of ask and dapA could increase the yield of FK506 by 18.28% and 23.14% respectively [12,14]. Mr. Li also confirmed this result [13]. After expressing a large number of dahp in shikimate pathway, the yield increased by 51.08%. This is by far the most effective way to change genes [11]. Ms Schulz [6] also combined precursor engineering with enzyme engineering: heterologous overexpression of the feedback-resistant aspartate kinase AskCg from Corynebacterium glutamicum, combined with overexpression of endogenous DapASt, enhanced FK506 production by approximately 73%; subsequent introduction of the PipAf Ile91Val mutant into this background achieved an approximately two-fold increase in yield over the wild type-expanding the upstream lysine pool while simultaneously enhancing downstream catalytic efficiency, thereby generating a "push-pull" synergistic effect.

We found that eliminating the genes of competing branches also had the same effect: after eliminating gcdh, the yield increased by 40.64% [12]. After turning off gdhA and ppc, the output increased by 37.77% and 32.26% respectively [11]. Mr. Wu [15] proved that if the redundant PKS pathway competing for the common accelerator is blocked, the metabolic process will develop in the direction of FK506, and the output will increase from 140.3 mg/L to 170.3 mg/L. Given that the targets of overexpression and knockout strategies are non-overlapping, combined schemes such as "overexpression of dahp plus knockout of gcdh" may produce super-additive synergistic effects.

As summarized in Table 1, exogenous precursor supplementation represents a rapid and effective supplementary approach.

Table 1. Effects of exogenous precursor supplementation.

Supplement	Concentration (g/L)	Yield improvement	References
L-Lysine	0.05–2.50	12.91%–190.00%	[14,16,17]
L-Pipecolic acid	0.25	11.81%	[16]
Shikimic acid	0.05–0.50	14.04%–52.99%	[14,16]

The effect of adding lysine varies with the strain. This shows that the bottleneck situation of each strain is different. In the strain whose ability to make lysine is very weak, adding lysine from the outside can make up for many shortcomings. The

things needed in the previous stage have become enough little by little. In the strain that can do a lot, the most bottleneck stage may have shifted to FkbL itself. Only a small improvement of 11.81% was achieved when pipecolic acid was directly added from the outside [16], indicating that the speed of metabolites entering from the outside passing through the membrane and whether they are not easily broken in cells may be another limitation.

4. MULTI-LEVEL REGULATORY ENGINEERING: FROM TRANSCRIPTION TO TRANSLATION

4.1 Overexpression and Multi-Copy Strategies of fkbN

Transport rules of fkbN gene manufacturing LAL-family transcriptional regulator. The regulator directly regulates most genes in the genome of FK506, such as fkbABC, fkbL, fkbD and fkbm [18]. Zhang et al. [19] used UV mutagenesis to obtain high-yield mutant strains and conducted Transcriptome-guided engineering. If another fkbN is added, the amount of FK506 will become 1,342 mg/L. Compared with the original stock, it increased by 290%. Interestingly, after replacing the original ermEp promoter with strong kasOp and stnk4p promoters, the yield decreased. It can be seen that the characteristics of the original promoter adjusting with time are very important to maintain the stability of metabolism.

4.2 Translational Optimization: A New Regulatory Dimension through Rare Codon Engineering

In 2025, Mr. Lee [5] published a paradigm-shifting multi-omics study that produced different ideas from the past. Combining genomics, differential RNA-seq, $ Term-seq, ribosome profiling (Ribo-seq), The particularly surprising discovery of s.tsukubaensis nrrl when the researchers made FK506 came from ribosome profiling analysis: encrypting TTA rare codon in allK gene, an important enzyme that produces allylmalonyl-CoA, would prevent ribosome, Until it can be clearly seen that TTA is only 0.01% in all codons of Streptomyces, due to the decrease of the expression of corresponding tRNA gene bldA, the force to read this codon drops sharply in the steady state. After replacing TTA codon with CTC, the stop of ribosome decreased by about 1/16, and the output of FK506 increased by 1.6 times. This discovery has changed the common sense so far, mainly improving metabolic engineering on transcription and precursor supply level. Let me understand that rare codon's stopping translation is a hidden problem in FK506 production so far. There are five TTA codon in FK506 gene cluster. If all the rare codon are optimized in a planned way, the yield may be further improved.

4.3 Indirect Regulatory Factors and Multi-Gene Synergy

FkbR/tcs7 always has a good regulatory effect in all strains, but the regulatory effect of fkbN in different strains may be opposite [20-22]. Xiao et al. [23] studied the homologous regulator fkbR2 in FK520 (ascomycin) mechanism, giving us a new view. If a lot of fkbR2 is added, the manufacturing capacity of FK520 will increase by 34%. However, according to the survey of electrophoretic mobility shift assays (EMSAS), protein of fkbR2 does not directly bind to the promoter regions of fkbO, fkbE, fkbU, fkbS, fkbL, fkbN, or fkbR1. From this discovery, it can be seen that FK506/FK520 may not directly stick to DNA, but work on the contact network between protein, and there are indirect regulatory factors that have not been well investigated.

Huang et al. [16] amplified five genes (fkbO, fkbL, fkbP, fkbD, fkbM, collectively referred to as fkbOLPDM box) together, and the yield of FK506 was 146.13%. This result shows that the construction of L-pipecolic acid, peptide skeleton connection and subsequent modification work are mutually restricted, and only when the three functions are blocked at the same time can the whole pathway flow. Taken together, a multi-tiered regulatory engineering hierarchy has emerged: multi-copy fkbN overexpression serves as “coarse-tuning” at the transcriptional level; multi-gene cassette overexpression provides “fine-tuning”; TTA rare codon replacement offers a third dimension of optimization at the translational level; and the systematic discovery of indirect regulatory factors opens new territory for future exploration.

5. FERMENTATION PROCESS OPTIMIZATION AND HIGH-THROUGHPUT SCREENING

5.1 Integrated Strategies: Carbon/Nitrogen Sources, Precursor Feeding, and Product Adsorption

Genetic engineering gives its strain the power to produce; The conditions of fermentation determine how much this force can actually produce. Yan et al. [24] optimized by response surface methodology, and adjusted the concentration of FK506 in shake flask to 1,293 mg/L and 1,522 mg/L in 1,000 L fermenter. Xia et al. [25] thought of adding several substances in stages, which increased the concentration of FK506 from 251 mg/L to 405 mg/L (an increase of 61.4%); The most important idea in this study is that the time period when the respective precursors can be added at the best time will change with the time when the corresponding metabolic pathways naturally start working when the cells grow.

Zhang [19] tried to further solve the feedback problem of the product after getting the high-yield strain 2108 n-1-4/DfkbN (1342 mg/L). We found that when the concentration of FK506 in the medium exceeded 500 mg/L, the growth of strain was obviously inhibited. In order to solve this problem, 20 g/L macroporous adsorption resin LX-60 was put into fermentation medium. As a result, 95% FK506 was adsorbed by resin, and the feedback inhibition of the product was well eliminated, and the titer rose to 2,663 mg/L (an increase of 98%). In addition, in order to improve the solubility of FK506 in water and the adsorption efficiency of resin, the titer of FK 506 in shaking flask was 3,746 mg/L after adding 35 g/L β-cyclodextrin. This is the highest value reported in the paper so far. The titer of the corresponding 5 L fermentor is 3,639 mg/L. The success of this combination method shows that compared with the supply of precursors, product feedback becomes a more urgent limiting factor in high-yield strain.

5.2 High-Throughput Screening to Accelerate Strain Development

Traditionally, when bacteria producing a lot of FK506 are selected, they should be fermented for 9 days and investigated by HPLC. Therefore, the number of bacteria that can be investigated at one time is very small. Mr. Cen [26] has worked out a quick selection method of using Saccharomyces cerevisiae growth inhibition zones: agar plugs of S. tsukubaensis colonies are placed face-down onto agar plates spread with yeast cells, and the FK506 yield is indirectly assessed by measuring the diameter of the growth inhibition zone. We not only tested both wild-type and calcineurin mutant (cnb1Δ) yeast strains, and supplemented the medium with different antifungal agents (fluconazole, amphotericin B, caspofungin), the inhibition zone diameter ratio between high- and low-yielding strains reached a maximum of 2.67 (wild-type yeast with fluconazole). It was found that many FK506 methods reduced the selection time from 9 days to 4 days, and HPLC was not needed. If this method is combined with metabolic engineering and fermentation optimization methods, the iteration of "transformation → screening → re-transformation" can be greatly accelerated.

6. CONCLUSIONS AND PERSPECTIVES

Focusing on the L-pipecolic acid supply bottleneck, this review has examined FK506 metabolic engineering strategies from four perspectives: FkbL catalytic properties, metabolic flux redirection, transcriptional and translational regulation, and fermentation optimization. The currently available evidence demonstrates that hierarchical transcriptional regulation combining multi-copy fkbN overexpression with multi-gene cassette engineering achieves the highest efficiency (146% improvement with the fkbOLPDM cassette; 290% improvement with dual-copy fkbN). Structure-guided rational enzyme design has provided proof-of-principle that the FkbL catalytic efficiency bottleneck can be effectively overcome (approximately 100% improvement with PipAf Ile91Val). Multi-omics integrative analysis has revealed a previously unrecognized regulatory dimension at the translational level (1.6-fold improvement by TTA→CTC codon replacement). The combined product adsorption and solubility optimization strategy has pushed FK506 titer to a record high of 3,746 mg/L [19].

Looking ahead, the following directions merit particular attention: (1) directed evolution of FkbL guided by AlphaFold2 structural models and the experimentally solved PipAf crystal structure, coupled with microfluidic ultrahigh-throughput screening or yeast-based inhibition zone screening [26] for efficient characterization of mutant libraries; (2) synergistic optimization at both transcriptional and translational levels, particularly the systematic replacement of all TTA rare codons within the FK506 gene cluster [5]; (3) systematic discovery of indirect regulatory factors (such as FkbR2 analogs) and multi-omics analysis of their regulatory networks [23]; (4) CRISPR-based editing tools [27] for the precise biosynthetic generation of FK506 structural analogs [28]; (5) systematic evaluation of non-food carbon sources such as crude glycerol and lignocellulosic hydrolysates for FK506 fermentation to address industrial sustainability requirements; and (6) automated synthetic biology platforms driving "design-build-test-learn" (DBTL) cycles that integrate metabolic engineering, fermentation optimization, and high-throughput screening into an iterative engineering pipeline [27]. With the progressive development of these methodological systems, the L-pipecolic acid bottleneck in FK506 biosynthesis may ultimately be overcome, advancing this essential immunosuppressant toward more efficient, lower-cost, and more sustainable industrial production.

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