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Lactococcus lactis as a Plasmid-Based Platform for Live Biotherapeutic Applications in Phenylketonuria: A Comprehensive Review

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Abstract:

Phenylketonuria (PKU) is an inherited metabolic disorder characterized by deficient activity of phenylalanine hydroxylase, leading to toxic accumulation of phenylalanine. Current therapies rely primarily on dietary restriction or enzyme substitution, but long-term compliance and systemic side effects remain challenges. Recent advances in synthetic biology and probiotic engineering have enabled the development of live biotherapeutic products (LBPs) capable of in situ metabolic correction. *Lactococcus lactis*, a Gram-positive, non-colonizing, and generally recognized as safe (GRAS) bacterium, has emerged as a promising chassis for plasmid-based delivery of therapeutic enzymes. This review explores the biological features of *L. lactis*, plasmid engineering strategies, mechanisms of gastrointestinal delivery, preclinical and clinical evidence supporting microbial therapeutics, biosafety and regulatory considerations, and future perspectives for PKU treatment. Emphasis is placed on plasmid-mediated expression of phenylalanine ammonia-lyase (PAL) and strategies to enhance luminal phenylalanine degradation while maintaining host safety. The review integrates recent findings and key studies over the past five years to highlight the translational potential of *L. lactis* in metabolic biotherapy.

Keywords: Lactococcus lactis, Phenylketonuria, Live biotherapeutic products, Plasmid engineering, Phenylalanine ammonia-lyase, Synthetic biology

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive disorder resulting from mutations in the PAH gene, leading to impaired conversion of phenylalanine (Phe) to tyrosine (1–3). Elevated Phe levels are neurotoxic, causing intellectual disability, seizures, and behavioral disturbances if untreated (2, 3). Current management includes strict dietary Phe restriction, tetrahydrobiopterin (BH4) supplementation, or enzyme replacement therapy (e.g., pegvaliase) (4, 5). However, adherence is challenging, and systemic enzyme therapies can trigger immune responses.

Synthetic biology approaches have enabled the development of engineered probiotics as metabolic sinks, capable of degrading Phe within the intestinal lumen before systemic absorption (6–10). *Lactococcus lactis* (L. lactis), widely used in dairy fermentation, offers safety advantages, low immunogenicity, and established mucosal delivery records, making it an attractive chassis for plasmid-

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based therapeutic enzyme expression (11–18). Unlike Gram-negative hosts, *L. lactis* produces minimal endotoxin and transiently colonizes the gut, limiting horizontal gene transfer risks (19–23). This review provides a comprehensive evaluation of *L. lactis* as a plasmid carrier for PKU therapy, highlighting molecular tools, plasmid strategies, preclinical and clinical evidence, biosafety, regulatory frameworks, and future directions.

Phenylketonuria and Therapeutic Rationale for Microbial Intervention

Pathophysiology of PKU

Phenylketonuria (PKU) arises from mutations in the PAH gene encoding phenylalanine hydroxylase (PAH), the key enzyme converting phenylalanine (Phe) to tyrosine. Impaired PAH activity leads to systemic accumulation of Phe, which crosses the blood–brain barrier and disrupts neurotransmitter synthesis, including dopamine, norepinephrine, and serotonin (1–3). Elevated Phe interferes with protein synthesis, causes oxidative stress, and affects myelin formation, ultimately resulting in cognitive deficits, motor dysfunction, and psychiatric manifestations if untreated (2, 3). The severity of PKU varies with the residual enzymatic activity: classical PKU (<1% PAH activity) requires strict dietary intervention, whereas mild hyperphenylalaninemia may be managed with less restrictive approaches (2). Understanding these molecular and neurological consequences underlines the need for effective, non-invasive therapeutic strategies capable of controlling plasma Phe.

Limitations of Current Therapies

Current management primarily relies on lifelong dietary restriction of Phe-rich foods such as meat, dairy, and legumes. While effective in reducing plasma Phe levels, strict diets are socially restrictive, nutritionally challenging, and difficult to maintain over time, especially in adolescents and adults (4). Pharmacological interventions, including BH4 (sapropterin dihydrochloride), can enhance residual PAH activity in responsive patients but are ineffective in the majority of classical PKU cases (4, 5). Enzyme replacement therapy with pegvaliase provides systemic Phe degradation; however, it requires parenteral administration and can trigger immune reactions, including anaphylaxis and antibody formation (5). These limitations highlight the clinical need for innovative therapies that offer consistent Phe control, minimal systemic exposure, and improved patient compliance.

Microbial Therapeutics as Metabolic Sinks

Engineered microbial therapeutics have emerged as promising alternatives, utilizing gut-resident or transiently colonizing bacteria to act as in situ metabolic sinks for Phe (6–10). By expressing enzymes such as phenylalanine ammonia-lyase (PAL) or L-amino acid oxidases, these microbes can degrade dietary and endogenous Phe within the gastrointestinal lumen before systemic absorption. Preclinical studies using PAL-expressing *E. coli* Nissle or *L. lactis* models have demonstrated significant reductions in plasma Phe, improved growth, and normalization of neurobehavioral parameters in PKU mice (8–10) (Table1). Microbial therapeutics offer advantages over systemic enzymes by targeting the site of absorption directly, potentially reducing immune reactions and improving patient quality of life.

Overview of Lactococcus lactis as a Therapeutic Chassis

Safety and GRAS Status

Lactococcus lactis is a non-pathogenic, Gram-positive, facultative anaerobic bacterium widely used in dairy fermentation and probiotic formulations. Its designation as GRAS by the FDA and long history of human consumption establish a strong safety profile (18–23). Unlike many Gram-negative bacteria, *L. lactis* produces negligible endotoxins, reducing the risk of inflammatory responses in the gastrointestinal tract. Clinical studies deploying *L. lactis* for cytokine delivery, such as IL-10 for Crohn's disease, have confirmed the organism's tolerability, even in vulnerable patient populations (23). Transient colonization of the gut further limits horizontal gene

Table 1. Comparison of key features between Lactococcus lactis and Escherichia coli as bacterial hosts for therapeutic applications

Feature	Lactococcus lactis	Escherichia coli	Explanation/Significance
Safety Status (GRAS)	Yes	No	L. lactis has a higher safety profile
Endotoxin Production	Minimal	High	Lower endotoxin reduces immune-related risks
Genetic Engineering Amenability	High	High	Both have strong genetic toolkits
Plasmid Types Used	Theta-replicating, Rolling-circle	Rolling-circle	Differences in plasmid stability and copy number
Suitability for Live Therapeutics	Yes	Limited	L. lactis is more Suitable for live biotherapeutic products
Colonization Duration	Transient	Transient	Both typically show temporary colonization

transfer and environmental persistence, making it ideal for live biotherapeutic applications.

Genetic Amenability

The genome of *L. lactis* has been fully sequenced, enabling precise genetic manipulation. Numerous plasmid vectors are available, including theta-replicating and rolling-circle plasmids, which differ in copy number, stability, and size capacity (24–27). Inducible systems such as the nisin-controlled expression (NICE) system provide tight regulation of heterologous gene expression, allowing high-level production of therapeutic enzymes while minimizing metabolic burden (25). Moreover, secretion and surface-display signals can be engineered to enhance extracellular activity of enzymes like PAL, enabling efficient interaction with luminal Phe.

Advantages Over Gram-Negative Hosts

Compared to conventional Gram-negative hosts such as *E. coli*, *L. lactis* has several advantages. It lacks lipopolysaccharide (LPS) endotoxin, reducing immunogenicity, and can be used without the risk of endotoxin contamination in clinical formulations (21). Additionally, *L. lactis* can be engineered with food-grade markers, avoiding the use of antibiotic selection, which is critical for regulatory approval of live biotherapeutics. Its safety, combined with genetic versatility and transient colonization, positions *L. lactis* as an optimal chassis for mucosal delivery of plasmid-based therapeutics.

Plasmid Engineering Strategies

Replicons and Copy Number

Plasmid choice significantly impacts gene expression, stability, and host fitness. Theta-replicating plasmids exhibit superior structural stability and low segregation loss, making them suitable for long-term therapeutic applications (27, 29). Rolling-circle plasmids, although capable of high copy numbers, may impose metabolic burdens on the host and reduce viability over time (26). Optimal plasmid design balances high-level enzyme expression with minimal impact on bacterial growth and survival in the gut.

Food-Grade Selection and Containment

Traditional antibiotic resistance markers are unsuitable for clinical applications due to regulatory and safety concerns. Food-grade selection systems, including auxotrophic complementation, lactose or amino acid dependence, provide selective pressure without antibiotics (31–32). Biocontainment strategies, such as kill-switches, quorum-sensing regulation, and metabolic dependencies, prevent uncontrolled proliferation and horizontal gene transfer in the environment (33).

DNA Delivery (Bactofection)

Beyond enzyme expression, *L. lactis* can deliver plasmid DNA directly to mammalian epithelial cells, a process termed bactofection (34–35). This approach expands therapeutic options by allowing transient expression of enzymes or regulatory proteins in host cells. Surface display of adhesion molecules, such as fibronectin-binding proteins, enhances plasmid uptake and intracellular delivery, providing a versatile platform for gene-based therapies.

Mechanisms of Plasmid-Mediated Therapeutic Delivery

Intestinal Phenylalanine Absorption

Dietary Phe is primarily absorbed in the small intestine through neutral amino acid transporters, such as LAT2 (36). The rapid absorption kinetics necessitate highly efficient luminal degradation to prevent systemic Phe accumulation. Engineered *L. lactis* can establish a local metabolic sink, intercepting Phe before it enters the bloodstream.

Intracellular vs Extracellular Enzyme Expression

Intracellular PAL expression confines degradation to bacterial cytoplasm, requiring uptake of Phe into the cell, potentially limiting efficiency. In contrast, extracellular or surface-anchored PAL allows direct enzymatic interaction with luminal Phe, significantly enhancing degradation rates (37–38). Secretion systems or cell-wall anchoring motifs can be optimized to maintain enzyme stability in the gastrointestinal environment.

Localized Metabolic Sinks

The concept of a luminal metabolic sink leverages the transient colonization of *L. lactis* in the small intestine. By expressing PAL at sufficient levels, these bacteria convert Phe into trans-cinnamic acid and ammonia, which are excreted (39–40). Modeling studies suggest that even partial degradation of dietary Phe in the lumen can significantly reduce systemic Phe levels, highlighting the therapeutic potential of this approach.

Engineering L. lactis for Phenylalanine Degradation

PAL Expression in Probiotics

PAL-expressing strains of *E. coli* Nissle have been shown to reduce plasma Phe in PKU mouse models, validating the concept of microbial Phe degradation (40–41). Translating this strategy to *L. lactis* requires careful optimization of codon usage, promoters, and secretion signals to achieve sufficient expression while maintaining bacterial fitness (42). Comparative analyses indicate that *L. lactis* may offer advantages in immunogenicity and safety over Gram-negative

hosts.

Host Fitness Considerations

High-level heterologous expression can impose metabolic stress, reducing growth rate and viability (42). Systems biology approaches, including transcriptomic and metabolic flux analyses, can identify bottlenecks and optimize expression systems. Dynamic promoters and feedback-regulated circuits allow PAL expression to respond to luminal Phe concentrations, balancing therapeutic activity with host survival.

Synthetic Biology Approaches

Advanced synthetic biology tools enable sophisticated control over enzyme expression. CRISPR/Cas systems can edit the genome to optimize metabolic pathways, while synthetic promoters responsive to Phe levels allow adaptive PAL production (33, 43). Integration of biosensors and logic circuits can further enhance therapeutic precision and safety.

Preclinical and Clinical Evidence

Engineered microbial therapeutics have demonstrated efficacy in preclinical models and are advancing into clinical evaluation. SYNB1934 and SYNB1618, PAL-expressing engineered probiotics, reduce plasma Phe in adult PKU patients and show acceptable safety profiles (8–10), (44–45). Clinical studies with *L. lactis* platforms (e.g., AG013, AG019) confirm the feasibility of mucosal delivery, safety, and stability of live biotherapeutic products (11, 51). These studies provide strong evidence for translational potential (Diagram).

Diagram: Mechanism of *Lactococcus lactis* Action in Reducing Phenylalanine Levels in PKU

1.Oral administration of engineered *Lactococcus lactis*

↓

2.Transient colonization in the small intestine lumen

↓

3.Expression and secretion (or surface display) of Phenylalanine ammonia-lyase (PAL) enzyme

↓

4.PAL enzymatically converts Phenylalanine (Phe) into trans-cinnamic acid and ammonia

↓

5.Reduced luminal Phenylalanine absorption into the bloodstream

↓

6.Excretion of metabolic products (trans-cinnamic acid and ammonia) via feces and urine

Diagram illustrates the therapeutic mechanism by which engineered *Lactococcus lactis* degrades luminal Phenylalanine to lower systemic Phe levels in phenylketonuria (PKU) patients, acting as a metabolic sink within the gastrointestinal tract.

Biosafety and Regulatory Considerations
Biocontainment

Ensuring environmental and patient safety is critical. Kill-switch circuits, auxotrophic designs, and quorum-sensing regulation prevent uncontrolled proliferation and horizontal gene transfer (33, 49). Immunocompromised patients require careful assessment of risk before administration (48, 51).

Regulatory Frameworks

LBPs are regulated as biological products by agencies including FDA and EMA. Regulatory requirements include quality control, genetic stability, preclinical safety, and phased clinical evaluation (45–46, 52). Post-market surveillance ensures ongoing safety and efficacy (53).

Future Perspectives
Advanced Genetic Tools

CRISPR-based regulatory circuits, biosensors, and dynamic promoters enable precise spatiotemporal control of PAL expression, enhancing safety and therapeutic efficacy (54–57).

Personalized Therapies

Patient-specific microbiome analysis and metabolic profiling allow optimization of microbial therapeutics for individual Phe metabolism patterns, improving efficacy (58–59).

Integration With Non-Living Biotherapeutics

Hybrid approaches using inactivated microbes retain enzyme activity while further reducing biosafety concerns, providing an alternative strategy for vulnerable populations (60–61).

9.4 Expansion to Other Metabolic Disorders

Strategies developed for PKU can be adapted to treat urea cycle disorders, hyperoxaluria, and branched-chain amino acid disorders, demonstrating broad applicability of engineered microbial therapeutics (62–65).

DISCUSSION

Engineering *L. lactis* as a plasmid carrier provides multiple advantages: safety, GRAS status, low immunogenicity, and amenability to advanced synthetic biology. The main challenges include balancing enzyme expression with host fitness, ensuring in vivo stability, and navigating regulatory pathways. Preclinical and clinical studies highlight potential for live biotherapeutics to complement

or replace current PKU therapies. Integration of biosafety circuits and personalized approaches will be critical for future success.

CONCLUSION

Lactococcus lactis offers a promising platform for plasmid-mediated delivery of therapeutic enzymes in PKU. Advances in synthetic biology, plasmid engineering, and metabolic modeling support its translational potential. Ongoing preclinical optimization and clinical studies, coupled with robust biosafety and regulatory frameworks, will be essential to realize its full therapeutic potential.

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