

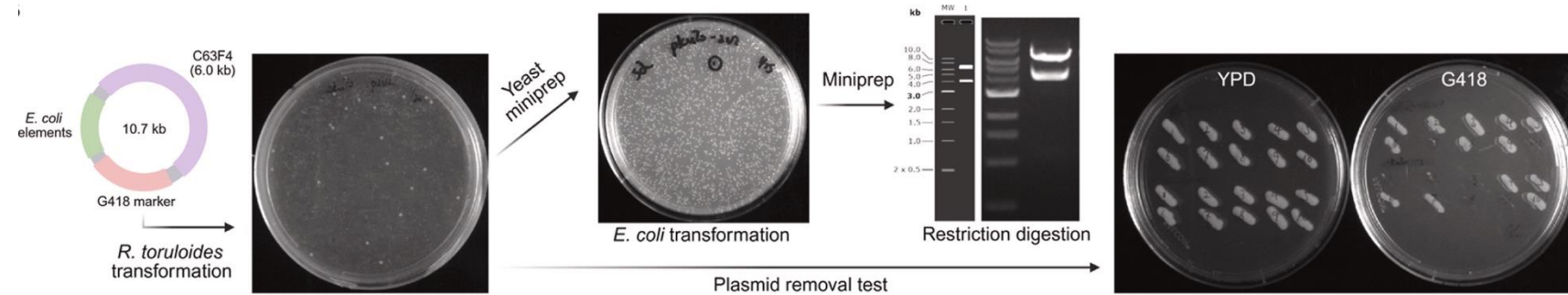
Development, Optimization, and Application of an Episomal Plasmid System for *Rhodotorula toruloides*

Background/Objective

Rhodotorula toruloides is an emerging oleaginous yeast with strong potential as a microbial cell factory for the production of acetyl-CoA-derived bioproducts. However, engineering of this organism has been limited by the absence of a functional episomal plasmid system, a foundational genetic tool for rapid gene expression, pathway testing, and CRISPR-based genome engineering. This study reports the first episomal plasmid system for *R. toruloides*.

Approach

Candidate autonomously replicating sequences from a variety of sources were systematically screened, and the sequence with the most stable performance was selected for detailed characterization. To overcome poor transformation efficiency, a Cre-loxP-mediated *in vivo* re-circularization strategy was developed to enable reliable delivery of the episomal plasmid.



Representative successful result of ARS screening for episomal plasmid development, using the pC63F4 plasmid as an example.

Results

C63F4, a fragment derived from *R. toruloides*, was selected because of its stable performance. The resulting pC63F4 plasmid was maintained episomally, supported GFP reporter expression, exhibited a copy number of 2.39 ± 0.13 , and showed good stability during long term cultivation. Researchers demonstrated functional episomal expression of metabolic engineering genes and multi-gene pathways for the triacetic acid production of triacetic acid lactone, fatty alcohols, and limonene. The platform was then leveraged to establish a redesigned CRISPR system that enables seamless genome editing in *R. toruloides* for the first time, while also simplifying marker recycling.

Significance/Impacts

This work establishes a long-needed episomal plasmid platform and associated CRISPR toolkit that will accelerate metabolic engineering, synthetic biology, and fundamental studies in *R. toruloides*.

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