A DNA assembly kit to unlock the CRISPR-Cas9 potential for metabolic engineering

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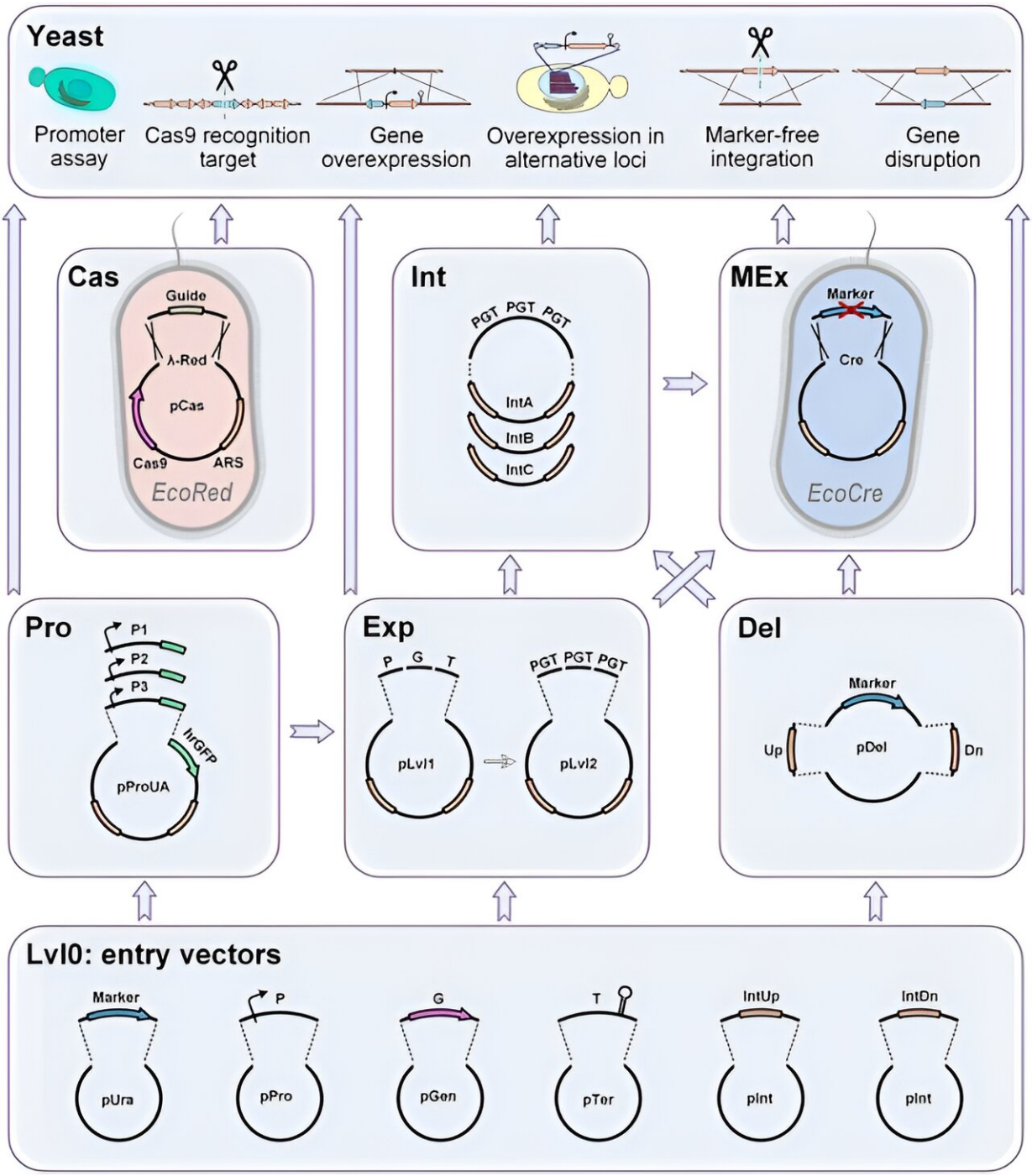


Modular toolkit structure - The toolkit consists of seven modules for quick and easy assembly of integrative constructs and Cas9-helper plasmids. Lvl0 Module: single parts in entry vectors. Exp Module: assembly of overexpression constructs. Pro Module: assembly and screening of new promoters. Del Module: assembly of disruption constructs. Int Module: changing integration loci by homology arms

exchange. MEx Module: assembly of marker-free constructs by selectable marker excision. Cas Module: redirection of Cas9-helper to new genome loci. Five of the modules - Pro, Del, Cas, Int, and MEx Modules - represent a new methodology that functionally extends previously used GG assembly systems. Assembly of Pro, Del and Int Modules are based on single GG reactions, while Cas and MEx Modules involve homologous and site-specific recombination taking place in the special E. coli strains. The arrows between modules indicate different orders in which they can be applied to enable variable genome engineering techniques as shown in the top panel, Yeast. Credit: *Nature Communications Biology*, doi: https://doi.org/10.1038/s42003-023-05202-5

The clustered regularly interspaced short palindrome repeats (CRISPR) and Crispr-associated protein 9 (CRISPR/Cas9) is now a well-known, revolutionary method to engineer microbial cells.

A key advantage of CRISPR remains in the strain design to facilitate chromosomal integration to enable the assembly of marker-free DNA. These editing systems are highly beneficial; however, their assembly is not quite straightforward and may prevent its use and applications.

In a new report in *Nature Communications Biology*, Tigran V. Yuzbashev and a research team identified the limits of the existing Cas9 toolkits designed to make CRISPR techniques easier to access and implement. They discussed three different well-established methods and combined them to form a comprehensive toolkit for efficient metabolic engineering by using CRISPR/Cas9.

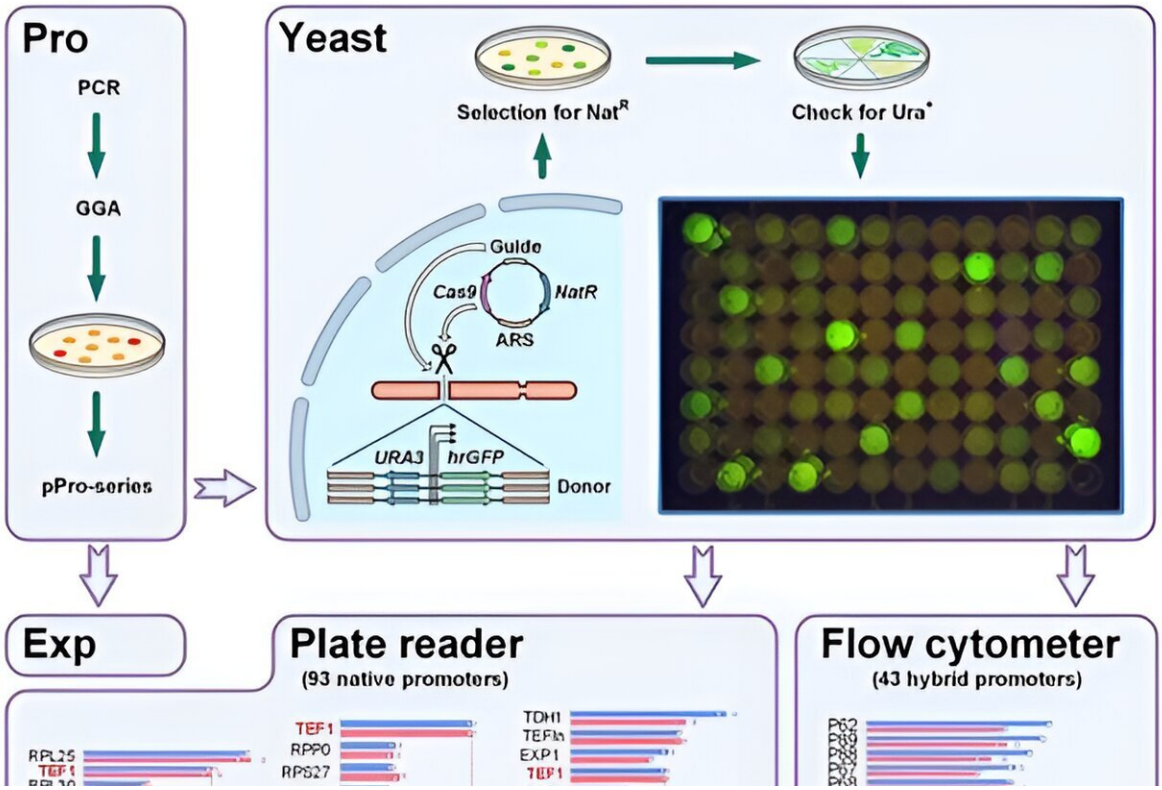
A single toolkit comprised of 147 plasmids to generate and characterize a library of 137 promoters to build a homogentisic acid in the lab.

Genome modifications with CRISPR/Cas9

The CRISPR/Cas9 system can render quick, precise and scarless genomic modifications to provide significant scope to design microbial strains for bioproduction. Metabolic engineering of yeasts for instance provide a fast-growing area in engineering biology for the sustained production of chemicals, fuels, foods, and pharmaceuticals.

Yeasts have a metabolic potential like eukaryotic cells and are therefore easier to engineer and cultivate at scale. As a result, bioengineers have designed and developed CRISPR systems for yeasts.

Due to its high efficiency, CRISPR allows marker-less genomic modifications. In this work, Yuzbashev ensured strain optimization and facilitated metabolic engineering projects by identifying three improvements of the CRISPR/Cas9 system for yeast engineering. The methods included: 1) the easy swap between marker and marker-less modifications, 2) the quick exchange of homology arms to determine different locations of integration, and 3) an easy method to clone gRNAs .



Promoter library screening using CRISPR/Cas9. In the Pro Module an amplified promoter can be easily assembled in a single GG step using RFP dropout selection. Resultant Pro-series plasmids can be used for both TU assembly in the Exp Module and for promoter assay after integration into the yeast genome. Single-copy integration into the standard locus is facilitated by a co-transformed Cas9-helper plasmid. Transformants are first selected by Nat-resistance encoded by the episomal helper, followed by Ura$^+$ phenotype verification confirming integration of the construct. The 96-well plate pictured demonstrates fluorescence of the Y. lipolytica transformant library with 93 native promoters grown in YPD medium (Section 8.1 of Supplementary material). Two bottom panels contain bar charts with 93 native and 43 hybrid promoters that were assayed using plate reader and flow cytometer respectively. Data were blanked using the parent strain S234 (0%) and normalized by TEF1 promoter activity (100%). For each promoter the GFP florescence was assayed in minimal (blue) and rich (red) media with either 2% glucose (YNBD, YPD) or 2% glycerol (YNBG and YPG) as the carbon source. Credit: *Nature Communications Biology*, doi: https://doi.org/10.1038/s42003-023-05202-5

Marker-free integration

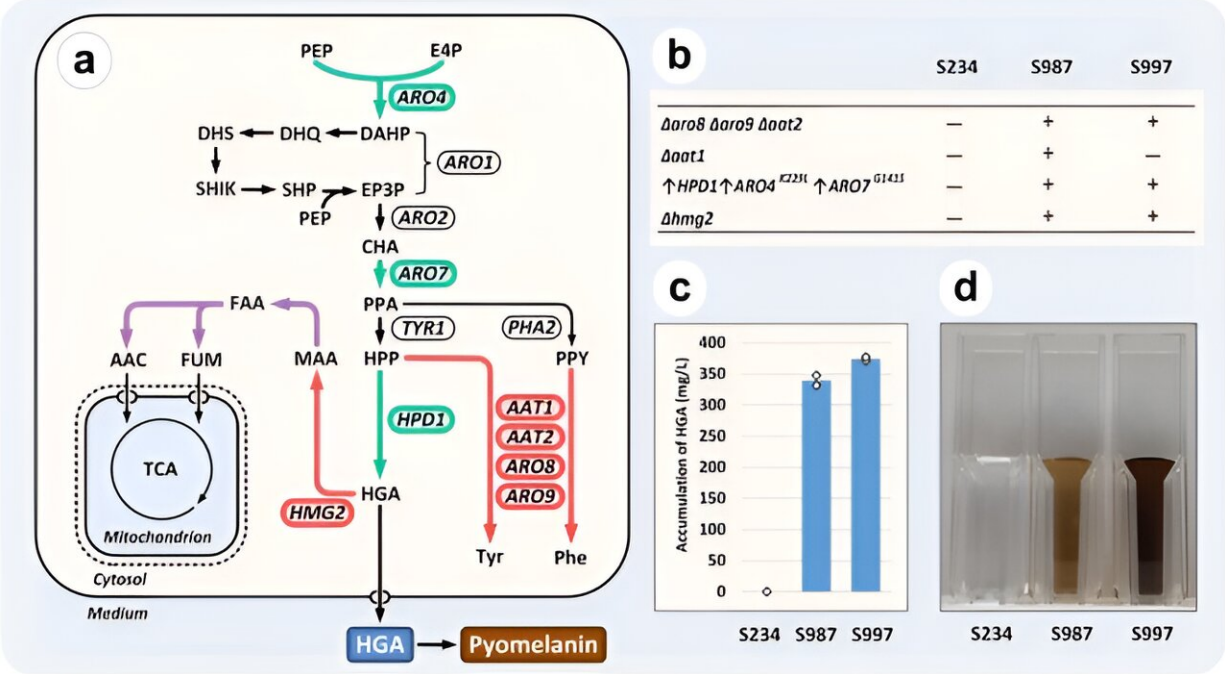
To enable CRISPR-based marker-free integration, the team chose a double strand break induced by Cas9, which had to be repaired to accomplish cell proliferation. The scientists made this possible by using a template or donor, integrated through homologous recombination or non-homologous end-joining (NHEJ)—without integration. The process of non-homologous end-joining is observed in most fungal species including baker's yeast Saccharomyces cerevisiae.

In species with a predominant NHEJ mechanism, the team enhanced homologous recombination by deleting the NHEJ genes. If a marker-free method did not succeed, the scientists subsequently aim to improve CRISPR-Cas9 assisted integration to easily revert to marker-based integration.

Donor DNA re-direction

The Cas9-assisted integration typically requires a donor template consisting of an integrated cassette flanked by two homology arms. The team theorized that the ideal integration of CRISPR/Cas9 should exchange homology arms on pre-assessed Cas9 donor constructs via a simplified Golden Gate assembly reaction.

Furthermore, promoters are a key element to any metabolic engineering project to redirect flux towards the products of interest. Yuzbashev et al. used the industrial yeast Yarrowia lipolytica to develop a metabolic engineering toolkit that combined gene editing and DNA assembly strategies for high efficiency and versatility.



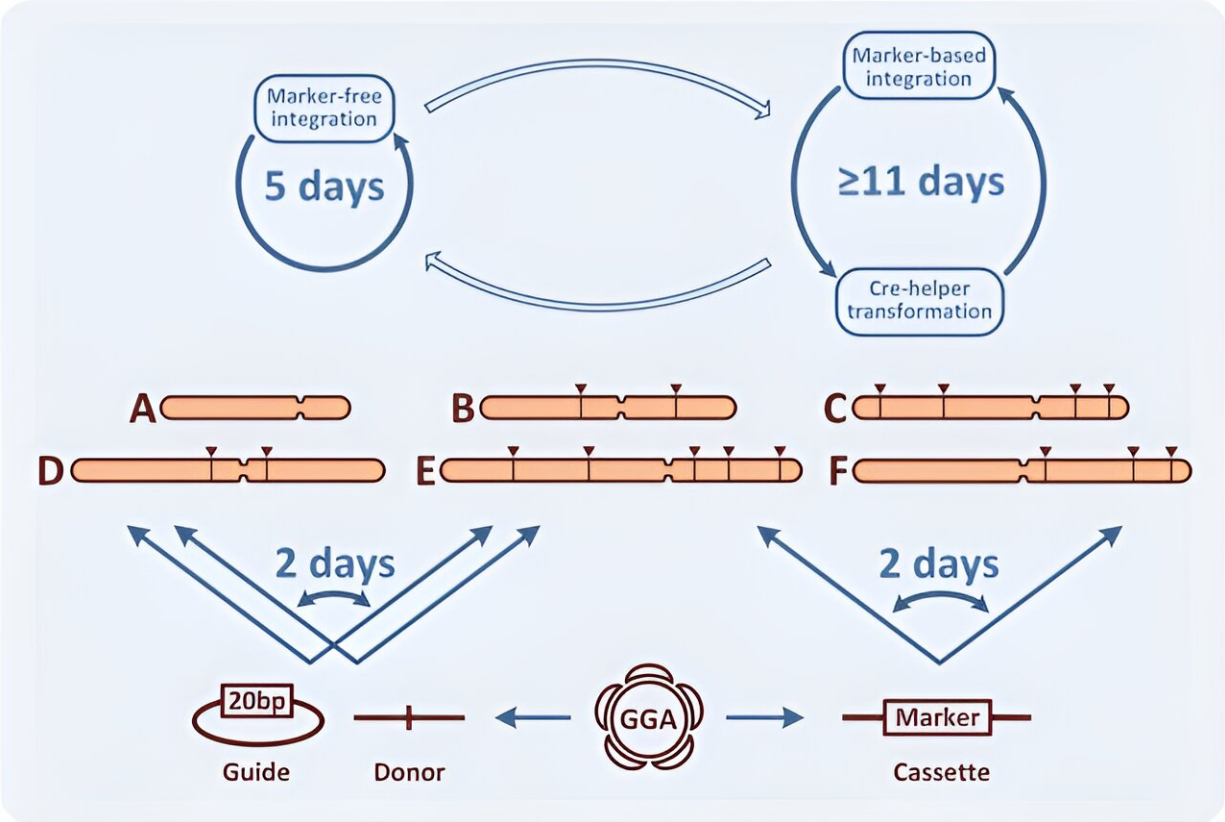
Validation of the toolkit for metabolic engineering. a) Schematic representation of the HGA producing pathway engineered in Y. lipolytica. Green arrows, overexpressed steps. Red arrows, inactivated reactions. Purple arrows, enzymatic reactions thought to be encoded by two other ORFs in the Y. lipolytica HGA degradation cluster. Genes encoding corresponding enzymatic steps are shown next to the reactions. Metabolites are shortened as follows: PEP phosphoenolpyruvate, E4P erythrose-4-phosphate, DAHP 3-deoxy-D-arabino-heptulosonate 7-phosphate, DHQ 3-dehydroquinate, DHS 3-dehydro-shikimate, SHIK shikimate, SHP shikimate-3-phosphate, EP3P 5-enolpyruvyl-shikimate-3-phosphate, CHA chorismate, PPA prephenate, HPP para-hydroxy-phenylpyruvate, PPY phenylpyruvate, MAA 4-maleyl-acetoacetate, FAA 4-fumaryl-acetoacetate, FUM fumarate, AAC acetoacetate, Tyr L-tyrosine, Phe L-phenylalanine. b) Summary of modifications introduced in HGA producing strains. The symbols of delta and up arrow are used to indicate deletion and overexpression respectively of corresponding genes. c) Accumulation of HGA by engineered strains after 14-day cultivation in YNB medium with 9% glucose. Two biological replicates are shown. d) Visible accumulation of pyomelanin by engineered strains in the cultural broth after seven-day cultivation in the YNB medium with 2% citrate. Credit: *Nature Communications Biology*, doi: https://doi.org/10.1038/s42003-023-05202-5

Modular architecture of the toolkit and metabolic pathway engineering

The scientists unlocked the full potential of CRISPR/Cas9 for metabolic engineering by developing a toolkit expanding on previously well-known Golden Gate assembly systems. They tested the screening system by generating several promoter libraries. Yuzbashev et al. chose Y. lipolytica ribosomal genes encoding proteins of large and small subunits. They identified a variety of promoters with diverse strengths to expand the number of promoters for the same organism.

To prove the influence and use of the enhanced CRISPR/Cas9 method, the team created a Y. lipolytica via rational engineering to produce a homogentisic acid (HGA) . Typically under alkaline conditions HGA spontaneously undergoes oxidation to form self-polymerized pyomelanin ; an excellent constituent of natural sunscreens and cosmetics.

Despite its high commercial potential, existing methods to produce the acid precursor and pyomelanin product relied on the biotransformation of expensive aromatic amino acids. To facilitate metabolic engineering, the team therefore first selected several genes that encoded theprecursor aromatic aminotransferases as engineering targets. They then selected three overexpression targets to enhance the de novo synthesis of the homogentisic acid in the model organism. Finally, they studied and inactivated the HGA degradation pathway; a path yet unknown to exist in Y. lipolytica.



The principle of accelerated metabolic engineering using the CRISPR/Cas9 toolkit. The toolkit allows frequent switching between marker-based and marker-free integration combining the advantages of both technologies. More robust marker-based integration requires at least 11 days, since it includes the marker recovery procedure. Application of the marker-free integration with CRISPR/Cas9 enables a single integration round in five days (image, top). Any single GG assembly reaction could be used for production of vectors with and without marker. At the same time, using the HA exchange system, any assembled integration cassette for overexpression, regardless of its complexity, can be redirected to alternative loci of the genome in two days. Moreover, both gRNA and donor can be redirected to alternative loci just in two days (image, bottom left). Credit: *Nature Communications Biology*, doi: https://doi.org/10.1038/s42003-023-05202-5

Outlook

In this way, Tigran V. Yuzbashev and colleagues showed the dependence of metabolic engineering of living organisms on efficient DNA manipulation methods. This work presents an example of an enhanced molecular toolkit designed for CRISPR/Cas9-based metabolic engineering.

The scientists proved the functionality of the platform for both rapid strain construction and the characterization of a large library of promoters. They anticipate for this toolkit to have broader applications in strain engineering and in industry. The team envision for the Y. lipolytica model developed in this work to have overarching applications in other fields of biological engineering as well.

More information: Tigran V. Yuzbashev et al, A DNA assembly toolkit to unlock the CRISPR/Cas9 potential for metabolic engineering, *Communications Biology* (2023). DOI: 10.1038/s42003-023-05202-5

Guri Giaever et al, Functional profiling of the Saccharomyces cerevisiae genome, *Nature* (2002). DOI: 10.1038/nature00935

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