Yeast knockout library allows for efficient testing of genomic mutations for cell-free protein synthesis

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ABSTRACT

Cell-free protein synthesis (CFPS) systems from crude lysates have benefitted from modifications to their enzyme composition. For example, functionally deleting enzymes in the source strain that are deleterious to CFPS can improve protein synthesis yields. However, making such modifications can take substantial time. As a proof-of-concept to accelerate prototyping capabilities, we assessed the feasibility of using the yeast knockout collection to identify negative effectors in a Saccharomyces cerevisiae CFPS platform. We analyzed extracts made from six deletion strains that targeted the single deletion of potentially negative effectors (e.g., nucleases). We found a statistically significant increase in luciferase yields upon loss of function of GCN3, PEP4, PPT1, NGL3, and XRN1 with a maximum increase of over 6-fold as compared to the wild type. Our work has implications for yeast CFPS and for rapidly prototyping strains to enable cell-free synthetic biology applications.

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1. Introduction

Cell-free protein synthesis (CFPS), which was first used to decipher the genetic code,[1] has recently made a renaissance with improved protein production capabilities and cost economics.[2] CFPS has been particularly useful for proteins that are difficult to produce via standard in vivo methods (e.g., membrane proteins[3–5] and proteins harboring non-standard amino acids[6–10]), high-throughput screening,[11–13] and clinical manufacture of therapeutics.[13–19] Additionally, the ease of protein production makes way for rapid prototyping in synthetic biology with applications in genetic circuits,[20–22] metabolism,[23] promoter libraries,[24] and diagnostics,[25] among others. This process uses crude cell lysates, or extracts, which contain the necessary translational machinery (including ribosomes, tRNAs, and aminoacyl tRNA synthetases) to drive protein synthesis in vitro.[2] Substrates such as nucleoside triphosphates (NTPs) and amino acids are added to the extract along with salts and other factors to mimic the cellular environment. Once DNA encoding the protein of interest is added to the reaction, the product can be made in a matter of hours. Because the protein synthesis reaction now occurs outside the confines of the cell membrane, there are benefits including the ability to control the reaction components and conditions, as well as the ability to decouple cell growth from protein synthesis.[2] Enabled by these benefits, several systems have emerged based on the lysates of Escherichia coli,[26] Saccharomyces cerevisiae,[27] wheat germ,[11] insect cells,[28] Leishmania tarentolae,[29] Chinese hamster ovary cells,[30] and tobacco BY-2 cells,[31] among others.

In addition to containing the necessary elements for translation, crude extracts also contain many other enzymes that have the potential to positively or negatively affect protein synthesis. Examples of negative effectors could include enzymes responsible for degrading DNA and proteins, as well as using resources such as ATP

that could be otherwise directed toward translation. Manipulations to the extract enzyme composition have shown utility in *E. coli* and wheat germ extracts. In one illustration, changes to extract processing methods removed protein synthesis inhibitors, such as thionins, and ribonucleases from wheat germ extract and allowed for production of up to 4 mg/mL dihydrofolate reductase in a continuous exchange reaction.[32] Also, work in the *E. coli* system used genomic modifications to remove deleterious enzymes from cell-free reactions by deleting their corresponding genes from the source strain.[9,33–36] These deletions included knocking out enzymes to impair amino acid and nucleic acid degradation pathways. For example, in order to address cysteine degradation, Calhoun and Swartz deleted the gene encoding for glutamate–cysteine ligase, which increased the lifetime of measurable concentrations of cysteine from 15 minutes to over 3 hours.[35] Additionally, Michel-Reydellet et al. were able to stabilize linear DNA fragments by deleting endonuclease I.[34] In a different genomically recoded chassis strain, Hong et al. observed a four-fold improvement in protein synthesis yields for products harboring non-standard amino acids through the deletion of five nucleases.[9]

Recently, our lab has developed a novel CFPS platform in yeast that enables rapid protein expression from linear PCR templates.[27,37–40] In terms of CFPS systems, our yeast platform benefits from being a microbe, a common protein production chassis, and a model organism. However, the platform currently suffers from low batch CFPS yields. Based on previous work, we hypothesize that this is due to the expected presence of nucleases and proteases,[27] non-productive consumption of energy substrates such as ATP and other nucleotides,[38] and low rates of translation initiation.[27,38,39] We also note that unlike the *E. coli* system described above, yeast CFPS does not appear to suffer from amino acid substrate limitations.[38] Guided by the results for wheat germ and *E. coli* CFPS systems above, we hypothesized that deleting potential negative effectors in the chromosome of the yeast crude lysate source strain could improve CFPS yields.

Many tools have been developed for engineering yeast. In particular, well established tools exist for the simple genomic modification of yeast cells through homologous recombination,[41] and now also through the CRISPR system.[42] As a model organism, the entire genome of yeast has been sequenced[43] and all open reading frames have been characterized in a yeast knockout (YKO) collection.[44] The use of this collection of strains can bypass the time investment for making a number of candidate mutations to characterize open reading frames. In a typical lab workflow, constructing single mutations in yeast takes approximately 7 days including primer design, PCR-based template construction, and knockout confirmation.

Here, our goal was to develop a method to rapidly test lysates from a series of single deletion strains in the YKO strain library, in order to efficiently identify gene deletions that can increase yeast CFPS yields. By leveraging the YKO collection, we aimed to reduce the total time for assessing a mutation by more than 50%. As a secondary objective, we wanted to assess the reproducibility of using the strains from the library along with our extract preparation methods. Thus, we set strict criteria for our work: the CFPS results came from two extracts of each mutant strain prepared from two separate fermentations.

We began by identifying possible negative effectors. Based on previous work primarily performed in yeast, but also *E. coli*, we targeted several relevant categories of enzymes. We chose knockouts of a protease (proteinase A, *pep4Δ*), two nucleases (poly-A specific exonuclease, *ngl3Δ*, and exoribonuclease, *xrn1Δ*), a phosphatase (protein phosphatase T, *ppt1Δ*), and two regulators of translation (eIF2 kinase, *gcn2Δ*, and eIF2B regulatory domain, *gcn3Δ*).[9,38,45] Proteinase A (Pep4) is one of two proteases responsible for approximately 86% of all protein degradation in yeast.[46] Next, given
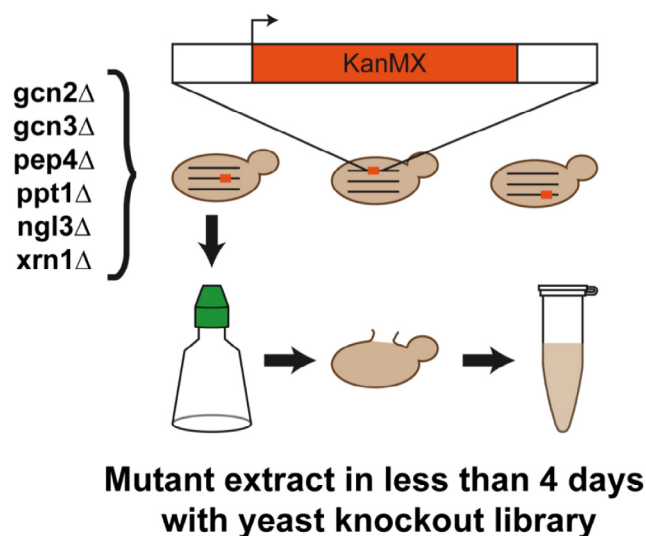

Fig. 1. A workflow diagram illustrates how the YKO collection is used for efficient screening of mutant strain extracts. Each nonessential ORF was deleted individually with the KanMX gene, shown here in orange, which imparts resistance to the drug G418. We selected a number of candidate strains from the yeast knockout collection that we subsequently grew in 1 L cultures, lysed, and processed into extract ready for use in cell-free protein synthesis.

the benefit seen in *E. coli* CFPS upon the deletion of nucleases,[9] as well as our reliance upon uncapped mRNA, we chose the strain deficient for exoribonuclease (Xrn1), a 5′-3′ exonuclease that acts on decapped mRNA. We also chose an exonuclease that acts in the 3′-5′ direction, Ngl3, which acts on polyA-RNAs. Protein phosphatase T (Ppt1) is a serine/threonine phosphatase. Based on previous work showing that our CFPS reactions are energy limited and that phosphate accumulates,[38] we included a phosphatase mutant to explore the possibility that phosphatase activity in the extract non-productively cleaves high-energy phosphate compounds in our cell-free reactions. Finally, given that translation initiation is considered the rate-limiting step in protein synthesis,[47] we chose the strains deleted for *GCN2* and *GCN3*, which are inhibitors of translation initiation. Gcn2 phosphorylates translation initiation factor eIF2α. When phosphorylated, eIF2α inhibits eIF2B through interaction with the eIF2B subunit, Gcn3.[48,49]

Strains harboring the above mutations were directly selected from the YKO library. As illustrated in Fig. 1, this library is a collection of yeast single mutant haploid strains, with each strain carrying one G418 resistance gene (KanMX) in place of every nonessential open reading frame of the S288c-derived BY4741. This is a commonly used laboratory strain that carries genetic auxotrophies (Supporting Information Table S1) to enable simple and fast genetic manipulation.[50] We used the MAT a collection because the original source strain we used for yeast CFPS, MBS, is MAT a.[27]

Next, we grew 1 L cultures of each of the yeast mutants in duplicate in order to prepare extract and assess variability (Fig. 1). Fermentations were harvested at mid-exponential phase with an average OD of 11.50 ± 0.76. Representative growth curves can be seen in Fig. 2A and B. Previous work has shown that yeast harvested at approximately 12 OD is the most productive for CFPS.[27,39] OD measurements were taken over the course of the fermentation and the growth rate was analyzed during exponential growth. The *gcn3Δ* growth curve is offset from the others. This is due to a lower starting OD, resulting in the delay in exponential phase. All strains except *gcn3Δ* had comparable growth rates, as assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test to compare each mutant to the wild type ($p < 0.01$).