INTRODUCTION

The central dogma of biology states DNA is transcribed to RNA, which is then translated to protein (Fig. 1) (1). As such, the sequence of DNA—or genotype—informs the proteins that are to be made, thus influencing phenotype. Genetic modification or mutagenesis of an organism genotype is one tool scientists use to investigate phenotype (1). One way to perform targeted mutagenesis is genome editing using Cas9, which is part of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system (2). In bacteria, CRISPR systems form a type of immune system, which defends the host by cleaving invading genetic material, such as viruses (3). Scientists are leveraging the power of CRISPR systems to edit the genomes of a wide variety of organisms. Application of this technology is fundamentally changing both industrial and academic science (4). To edit a genome with CRISPR, an investigator needs three things: 1) a nuclease that will cut the genome, most commonly Cas9; 2) a 20-base guide RNA (gRNA), which will guide Cas9 to the specific genome sequence using base pairing; 3) a repair template that, in coordination with the host DNA repair machinery, directs the repair of the double-strand break introduced by Cas9 and, in doing so, introduces a mutation (edit) to the genome (Fig. 2) (1).

The precision of this technique comes from the specificity of the 20-base guide RNA, which binds to complementary DNA bases at the target site. Since there are four possible bases in DNA (represented as A, G, C, and T), there are $4^{20}$ different 20-base pair sequence combinations. It is easy to change the sequence CRISPR targets by changing the sequence of the guide RNA. For cutting to happen at any given 20-base pair sequence, there is one additional requirement—the presence of a 3’ protospacer adjacent motif (PAM) sequence (Fig. 2). For Cas9, the PAM sequence is NGG, where N is any base (A, G, C, or T) (5, 6). After the Cas9/gRNA complex finds its corresponding DNA binding site, cleavage occurs on both DNA strands. Scissors indicate cleavage sites (Fig. 2). The cell’s DNA repair machinery uses the repair template to fix the double-stranded break. The repair template must disrupt the Cas9/gRNA nuclease recognition elements (gRNA binding site and/or PAM) for genome editing to occur. If the recognition elements were to remain, the nuclease could potentially bind and cleave the target sequence again.

PROCEDURE

Overview

In this exercise, students edit the genome of the budding yeast *S. cerevisiae* (7). People have been using yeast for thousands of years for making bread, wine, and chocolate—in recent years, it has also proven useful for the production...
of fuel ethanol and medicines (e.g., insulin). The described protocols use CRISPR to insert a stop codon into the ADE2 gene (a member of the adenine biosynthesis pathway). Mutations in ADE2 lead to the buildup of a precursor in the adenine biosynthesis pathway that turns the yeast a bright red color (Fig. 1). (It should be noted that while this exercise targets ADE2, any gene could be targeted by CRISPR in S. cerevisiae or a number of other yeast species by altering the guide and repair template sequences.) (8)

Transformation of S. cerevisiae

Grow overnight yeast extract-peptone-dextrose (YPD) culture of S. cerevisiae at room temperature, ideally to Optical Density 600 nm (OD$_{600}$) less than six. Serial dilutions of overnight cultures are helpful to ensure an overnight is at the appropriate OD when the lab period begins. Higher ODs will still likely transform but will be less efficient. Strains that work well for transformation include, but are not limited to, BY4741 or BY4743 strains classically used in yeast genetic laboratories, brewing strains available at homebrew stores and online (we have found Safbrew T-58, Lallemand Munich wheat, Lallemand Nottingham Ale, and Lallemand BRY-97 work well), and baking strains available at grocery stores and online (we have found Red Star Yeast works well). Multiple transformations with a variety of strains can be performed to compare transformation and gene editing rates between strains. Transform aliquots of yeast with either plasmid pV1382 (8), which encodes Cas9 and RNA guides, or plasmid pVG1 (8), which also contains repair templates designed to introduce stop codons to ADE2 (Fig. 3) (both plasmids are available through Addgene). Protocols to perform LiAc yeast transformations in under two hours, all reagent recipes, and product numbers are provided in the supplemental materials (Appendices 1, 2, and 3).

Plate each transformation on a separate petri dish containing appropriate media. If using ura- strains, such as BY4741, plate onto media lacking uracil Synthetic Complete–Uracil (SC-Ura). If using a prototrophic strain, such as a brewing or baking strain, plate onto YPD containing 100 μg/mL nourseothricin. URA3 and nourseothricin resistance (NatR) markers are encoded on the plasmid (Fig. 3). Edited colonies will appear and accumulate red color over the next two to seven days, while unedited colonies will remain white (Fig. 1). Students will count colonies and record their results during this time or next class. Suggested pre-lab exercises, learning objectives, post lab exercises, as well as all media recipes are provided in the supplemental materials (Appendices 1, 2, and 3).

Pedagogical aspects of activities-

Activities and assessments provided in Appendix 1 will guide students through the process of genetically engineering yeast. Reading the protocol and pre-lab questions will teach students the fundamentals of CRISPR-mediated genome editing in S. cerevisiae. Many of these core strategies are applicable to other model systems that are generally too complex to use in an undergraduate laboratory exercise. Furthermore, during the exercise, students will learn a number of microbiology techniques, such as transformation, that can be applied to analogous activities, for example transformation of other organisms. This allows a CRISPR transformation lab to replace or reinforce such labs in a curriculum. Finally, focused relevant discussions of genome editing during the incubation steps of this laboratory provide excellent opportunities for students and instructors to investigate current advances in genome editing. As CRISPR mediated genome editing is a rapidly developing field, such discussions will provide investigators and students an opportunity to explore cutting-edge technologies highly relevant to a wide variety of careers.
Student achievement is readily assessable using the materials provided in Appendix 1. For instance, pre-lab and post-lab reports and data tables can be collected and used to assess mastery. In addition, student participation during discussions could be used to assess understanding. However, the materials we provide are a guide, and we encourage instructors to edit these materials or create materials they feel are appropriate for their class to assess learning.

Tips for improving transformation

Although transformation with 5 μg of plasmid will, in most cases, give a number of correct transformants, additional plasmid can be used if more colonies are desired. Maxi and mega preps are excellent, cost-effective ways to generate large amounts of plasmid. In addition, allowing cells to recover after heat shock for longer can increase the number of transformants recovered. Finally, electroporation is often more efficient than heat shock transformation but requires special equipment. Protocols for electroporation can be found in Vyas et al. (8).

Safety issues

The experiments described in this paper genetically modify S. cerevisiae. In addition, plasmid must be purified for the experiments from E. coli. In the United States the work described is at Biosafety Level I (BSL1). If working in a different country, the laws guiding work with genetically modified organisms could differ and should be followed. Instructors and students should follow the ASM Biosafety Guidelines (https://www.asm.org/Guideline/ASM-Guidelines-for-Biosafety-in-Teaching-Laborator).

CONCLUSIONS

The above-described experimental design and supplemental materials provide students with the opportunity to gain an appreciation for how changes in genotype can lead to changes in phenotype while gaining experience in cutting-edge genome editing technology. The experiments offered are suitable for an introductory undergraduate or advanced placement (AP) high school biology course as they can be performed in two hours, do not require expensive equipment, are easily scalable for large classes, and provide easily interpretable results. These experiments can be embellished by having students design guide sequences or repair templates to edit any S. cerevisiae gene of interest.

SUPPLEMENTAL MATERIALS

Appendix 1: Laboratory exercises
Appendix 2: Media recipes
Appendix 3: Answer key

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