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"Optimizing CRISPR-Cas9 for Rhizopus delemar: sgRNA Screening via Bacterial On-Targeting Assays"

Rhizopus delemar, a zygomycetous fungus, is the main contributor to mucormycosis. Mucormycosis is a rare, but aggressive and life-threatening disease mainly affecting immunocompromised people, such as those with diabetes, cancer, or organ transplant recipients. The growing number of immunocompromised individuals has increased the incidence of mucormycosis. However, due to the high recalcitrant of R. *delemar* in genetic studies, much of its pathobiology remains uncharacterized. To improve genetic tractability, CRISPR-Cas9 technology was utilized for gene editing in R. delemar. We first attempted to identify single-guide RNA (sgRNA) with high-on-targeting efficiency. Using an innovative all-in-one noodle construct, we evaluated six sgRNAs selected by online ChopChop and WU-Crispr CRISPR tools that target the pyrF gene in R. delemar. We found that several sgRNA constructs exhibit various on-target efficiency during DNA replication in bacteria. This is interesting as the noodle constructs are designed to undergo sgRNA targeting in a mammalian host. To leverage the advantages of on-targeting in bacteria, we selected two sgRNAs and quantified their on-targeting efficiency in a bacterial host.

HB101 E. coli competent cells were transformed with plasmid constructs 122 and 226 (or C122 and C226), each containing a target sequence, donor template, and a carbenicillin resistance gene. Cells were heat shocked at 42 °C for 45 seconds, recovered in SOC medium, and plated on LB agar containing carbenicillin. Plates were incubated overnight at 37 °C. From each transformation, 10 single colonies were selected and inoculated into LB liquid medium with carbenicillin, then cultured overnight at 37 °C shaking at 225 rpm. Plasmid DNA was extracted using the Qiagen Plasmid Prep Kit. To assess plasmid integrity, each preparation was subjected to a double digestion with BamHI and EcoRI (1 µL each) in a 25 µL reaction using CutSmart buffer, incubated at 37 °C for 2 hours. Digestion products were analyzed on 0.7–1% agarose gels stained with DNA dye and visualized alongside a Versa DNA ladder. A non-transformed control plasmid served as the reference. We found that C226 showed no deviation from the control, whereas C122 samples showed a mix of complete and target-excised plasmids. Our study provides an invaluable tool to quickly identify sgRNA with high on-target efficiency that facilitates CRISPR-Cas9-mediated gene editing in R. delemar.