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Cas9 Endonuclease Toxicity in Haploid and Diploid Strains of *Saccharomyces cerevisiae*

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Abstract—Objective: CRISPR/Cas9-based RNA-guided genome-editing tools are highly effective. However, off-target mutations and cellular toxicity limit their utility. The main goal of the study is to investigate whether Cas9 exhibits toxicity towards budding yeast *Saccharomyces cerevisiae*. **Methods:** Haploid and diploid *S. cerevisiae* strains were engineered to express either Cas9 or both Cas9 and a single-guide RNA (sgRNA). Growth curve analysis was used to evaluate the impact of Cas9 expression on yeast viability. **Results and Discussion:** Haploid and diploid strains expressing both Cas9 and sgRNA exhibit lower growth rates compared to strains that do not produce either Cas9 or sgRNA, presumably due to effective double-strand breaks induction. Toxic effect of Cas9 without sgRNA was observed as well. Cas9 toxicity appears more severe in haploid yeast while diploid strain is more tolerant to Cas9 overproduction. Toxicity of Cas9 may be caused by its non-specific binding to genomic DNA or excessive protein production. **Conclusions:** The Cas9 endonuclease is toxic in *S. cerevisiae*. This may compromice the efficacy of the CRISPR/Cas9 editing system. Therefore, mitigating Cas9 toxicity should be one of the strategies for improving genome editing outcomes.

Keywords: genome editing, CRISPR/Cas9, Cas9 toxicity, genotoxicity

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INTRODUCTION

CRISPR/Cas9 is a convenient and efficient RNA-guided genome-editing platform. The Cas9 endonuclease, when bound to a single-guide RNA (sgRNA), introduces targeted double-strand breaks (DSBs) into genomic DNA. Subsequent changes at the target site arise via error-prone DNA repair mechanisms triggered by these breaks (Shumega et al., 2024). While the system offers notable benefits, such as ease of use and high editing efficacy, it has significant disadvantages. One of the most important concerns is genotoxicity, which can have detrimental effects on cells subjected to genome manipulation. Induced DSBs are hazardous lesions that can trigger cell cycle blockade and apoptosis (Friskes et al., 2022). Several studies have revealed correlations between off-target activity and Cas9-induced toxicity. For example, Morgens and coauthors documented reduced growth rates in K562 cells proportional to the extent of off-target DSBs formation (Morgens et al., 2017). Additionally, Laughery et al. proposed another mechanism where the Cas9/sgRNA complex triggers deamination of nitrogenous bases in ssDNA upon binding to chromosomal regions, further contributing to deleterious mutations (Laughery et al., 2019).

Some evidence suggests that Cas9 protein can be toxic to cells even in the absence of a sgRNA. For instance, Jacobs and colleagues (2014) showed that *Cas9* overexpression in fission yeast *Schizosaccharomyces pombe* causes intramolecular recombination within the *Cas9*-bearing plasmid that results in *Cas9* gene deletion and subsequent alleviation of its toxicity. Cas9 toxicity might arise from nonspecific interactions between Cas9 and genomic DNA during protospacer adjacent motif (PAM) searching. Moreover, Cas9-mediated toxicity appears influenced by GC content, as reported by Misra et al. (2019), who observed pronounced toxicities of the wild type and enzymatically inactive (dCas9)

protein in bacteria characterized by higher GC contents, *Mycobacterium smegmatis* and *Xanthomonas campestris*.

In this study, we examine the toxicity of the Cas9/sgRNA complex and Cas9 nuclease in both haploid and diploid *S. cerevisiae* strains.

MATERIALS AND METHODS

Yeast Strains

In this study, *Saccharomyces cerevisiae* LAN201 haploid strain (MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112) was used (Lada et al., 2013). LAN211 is an auto-diploid of LAN201 that was obtained through the expression of HO endonuclease, followed by selection for diploids.

Plasmids

The plasmid pML107, described by (Laughery and Wyrick, 2019) served as a backbone and the pESC-LEU-PmCDA1 plasmid (Lada et al., 2013) was utilized as the source of the *GAL1* promoter for the construction of three novel plasmids. The pML107-GAL1 plasmid expresses Cas9 alone, while pML107-GAL1-PC functions as an expression vector for the complete editing complex Cas9/sgRNA in a galactose-containing medium. The pML107-GAL1-ΔCas9 is a control plasmid lacking the Cas9 encoding sequence. Plasmids pML107-GAL1, pML107-GAL1-PC and pML107-GAL1-ΔCas9 will be further referred to as Cas9, Cas9-sgRNA and ΔCas9, respectively.

Growth Curve Plotting

Yeast strains LAN201 and LAN211 were transformed with the plasmids obtained in this study: pML107-GAL1-PC (Cas9-sgRNA), pML107-GAL1 (Cas9), and pML107-GAL1-ΔCas9 (ΔCas9). Transformants were selected on synthetic dropout SD medium (Sherman, 2002) lacking L-leucine (SD-leu), which contained 2% glucose and all necessary supplemental nutrients for the growth of LAN201 transformants, specifically adenine, L-lysine, L-histidine, and L-tryptophan. For the growth curve analysis, three independent clones of each transformant were utilized. A small volume of yeast cells was inoculated into 10 mL of liquid SD-leu medium. Subsequently, 2 mL of stationary-phase cultures were used to inoculate 50 mL of synthetic media devoid of L-leucine, supplemented with 2% galactose (MG-leu) in place of glucose, along with uracil, adenine, L-lysine, L-histidine, and L-tryptophan. Galactose activates the *GAL1* promoter, thereby inducing *Cas9* expression. The initial mean optical density at 600 nm (OD600) for Cas9-sgRNA, Cas9 and ΔCas9 transformants of haploid strain was 0.07, while for diploid one OD600 was 0.05. Over the course of 24 h, OD600 measurements were taken every two h, and growth curves were plotted.

We conducted a non-linear regression analysis of the plotted growth curves using a sigmoidal model. All curves exhibited an excellent fit to the selected model ($R^2 > 0.98$). Growth rate (r) for each culture was calculated using following formula (Hall et al., 2014):

$$r = \frac{\ln (OD_t / OD_0)}{t},$$

where OD_0 is OD600 at initial point ($t = 0$), $\underline{OD}_t$ is OD600 at time point t. Growth rates were used to estimate doubling time (DT), which is time (in h), required to double cell density of culture:

$$DT = \frac{\ln 2}{r}.$$

Since growth rate is not the same in different time point in sigmoidal model, for each transformant we defined the r in the point, where it is the highest (Toussaint et al., 2006). These time points for LAN211 (Cas9-sgRNA), (Cas9) and (ΔCas9) were 21.8, 18.0 and 16.8, respectively. For LAN201 (Cas9-sgRNA), (Cas9) and (ΔCas9) they were 22.3, 18.3 and 15.3, respectively.

To compare DT between transformants, 2-way ANOVA was performed with subsequent Tukey multiple comparisons test.

RESULTS

Plasmid Construction

The plasmid pML107-GAL1, harboring the *Cas9* gene controlled by the *GAL1* promoter, was constructed as follows. Parental plasmid pML107 was initially subjected to double-digestion with EcoRI and BamHI, the 7493-bp fragment was then ligated with another fragment containing the *GAL1* promoter, which was isolated from the pESC-LEU-PmCDA1 plasmid (Lada et al., 2013) after digestion with the same enzymes. The pML107-GAL1-PC plasmid, which co-expresses both Cas9 and sgRNA specifically designed for targeting the yeast *URA3* gene,

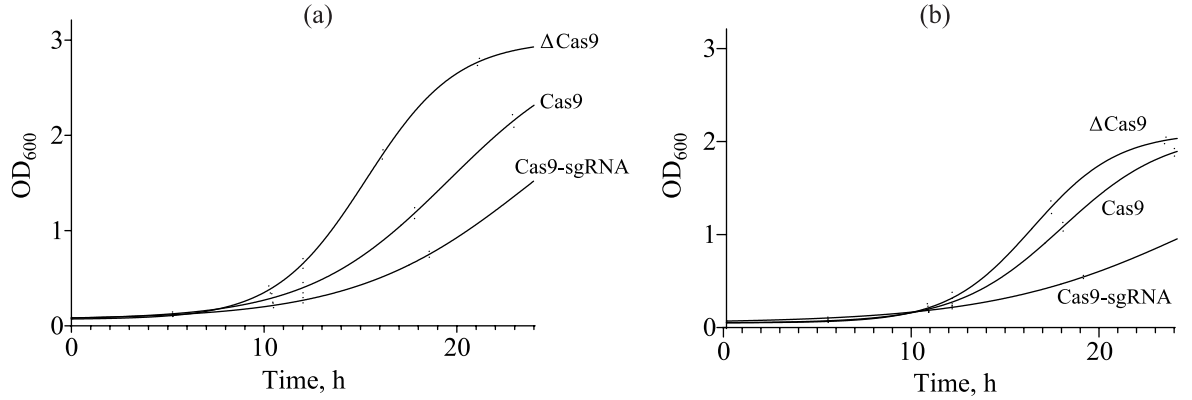

Fig. 1. Growth curves of (a) haploid and (b) diploid *Saccharomyces cerevisiae* strain transformed with Cas9-sgRNA, Cas9, and ΔCas9. Confidence intervals (CI 95%) are shown by the dotted lines.

was constructed by cloning of hybridized oligonucleotides (5′-GATCGATTGGTTGATTATGACACCG-TTTTAGAGCTAG-3′ and 5′-CTAGCTCTAAAA-CGGTGTCATAATCAACCAATC-3′, 20-nt guide sequence is underlined) into pML107-GAL1 according to the authors' recommendations (Laughery and Wyrick, 2019). The pML107-GAL1-ΔCas9 plasmid, which lacks both Cas9 and sgRNA sequences, was constructed as follows: pML107-GAL1 was digested with BamHI and NcoI, end-blunted and self-ligated to delete the *Cas9* gene.

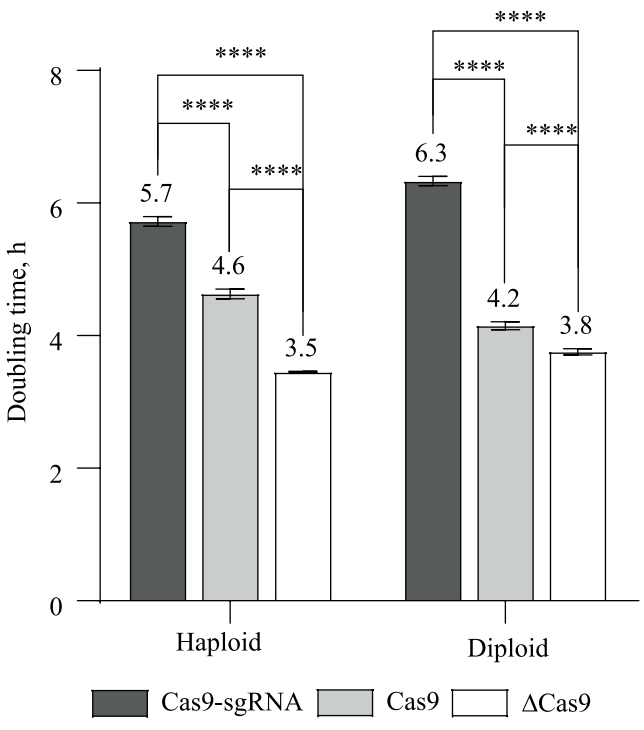

Fig. 2. Doubling time (DT) of haploid and diploid *Saccharomyces cerevisiae* strain transformed with Cas9-sgRNA, Cas9, and ΔCas9 plasmids. Standard deviation (SD) is shown as error bar. (****) p-value < 0.0001.

The Impact of Cas9 Expression on the Growth Rates of Haploid Yeast

The haploid *S. cerevisiae* strain LAN201 was transformed with either Cas9, Cas9-sgRNA or ΔCas9 plasmids. Transformants were subsequently cultured in MG-leu medium. OD600 readings were recorded over a period of 24 h, resulting in growth curves presented in Fig. 1a. DT of cells in each culture is shown in Fig. 2. Cas9-sgRNA transformant had the lowest growth rate (DT = 5.7) likely due unrepaired DSBs induced with Cas9/sgRNA complex. Conversely, ΔCas9 transformant demonstrated the highest growth rate (DT = 3.5). It reached deceleration phase by approximately 20 h of incubation while Cas9 and Cas9-sgRNA just entered exponential phase at that time point. The Cas9 transformant exhibited an intermediate growth rate (DT = 4.6). It was higher than the rate of Cas9-sgRNA transformant due to lack of DSBs, but lower than the growth rate observed for the LAN201 (ΔCas9). This indicates that Cas9 itself, even without forming a complex with sgRNA, imparts toxicity to haploid yeast.

The Impact of Cas9 Expression on the Growth Rates of Diploid Yeast

Diploid yeast strain LAN211, transformed with the same set of plasmids (Cas9, Cas9-sgRNA, and

ΔCas9), was analyzed similarly, with results presented in Figs. 1b and 2. First, we noticed, that there were notable differences in the OD600 of stationary phase between haploid and diploid strains. Diploid yeast cells tend to be larger than those haploid cells. This increased cell volume means that for a given number of cells, the total biomass may be greater in diploids, but the cell density (number of cells per unit volume) is lower, leading to a lower OD600 reading (Fukuda et al., 2023). Similar to haploid strain, the growth rate of diploid transformants with Cas9-sgRNA plasmid was significantly reduced compared to transformants carrying ΔCas9 plasmid (DT = 6.3 vs. DT = 3.8), indicating that the Cas9/sgRNA complex is toxic for diploid yeast. However, diploid yeast transformed with Cas9 exhibited less pronounced elongation in doubling time relative to those harboring ΔCas9, although this difference remained statistically significant (DT = 4.2 vs. DT = 3.8). Thus, while Cas9 endonuclease is toxic to diploid strain, but its toxic effect is milder than in haploid strain.

DISCUSSION

Analysis of haploid and diploid transformants expressing Cas9 reveals its toxicity in yeast *S. cerevisiae*. The primary cause of this toxicity is the generation of DSBs in genomic DNA by the Cas9/sgRNA complex. This damage triggers cell-cycle arrest at checkpoint stages, ultimately leading to apoptosis-like cell death (Burhans et al., 2003). However, the reasons behind the adverse effects of solely expressed Cas9 are more elusive. Cas9 appears to exhibit non-specific nuclease activity. This is supported by the fact that Cas9 is more toxic than its catalytically inactive counterpart, dCas9 (Misra et al., 2019). However, some authors consider Cas9 impossible to have nuclease activity without being in a complex with sgRNA (Lim et al., 2016). Moreover, Cas9's ability to sgRNA-independent DNA-cleavage was demonstrated only in specific in vitro conditions (Sundaresan et al., 2017). Excess positive net charge of Cas9 seems to result in its electrostatical interaction with genomic DNA and cellular RNA. Binding to chromosomal regions could disrupt essential processes, such as DNA replication and transcription, which leads to impaired cellular proliferation. We also suggest that toxicity of Cas9 endonuclease is caused by its overproduction in yeast cells. Constitutive production of such a large protein as Cas9 (1368 amino acid residues) on minimal galactose medium may affect yeast growth rate as a significant amount of nutrients is used for Cas9 synthesis.

Generally, heterologous protein expression in budding yeast causes metabolic stress reactions that reduces its growth rate (Mattanovich et al., 2004). The relatively greater tolerance of the diploid LAN211 strain compared to its isogenic haploid counterpart to Cas9 overproduction can likely be attributed to both lower levels of Cas9 protein production per unit cell volume and the presence of duplicate copies of all genes, including those that are essential for cellular viability.

CONCLUSION

Editing complex Cas9/sgRNA is equally toxic for haploid and diploid *Saccharomyces cerevisiae* strains. The Cas9 endonuclease solely is toxic for both haploid and diploid yeast as well, but its negative effect is more pronounced in haploid strain. Toxicity can lead to decreased transformation efficiency and, ultimately, low-yield genome editing. Since efficiency is one of the main challenges of genome editing, mitigating Cas9 toxicity represents an attractive area for further investigation.

ABBREVIATIONS AND NOTATION

DSB—double strand break;

DT—doubling time;

MG—minimal galactose (medium);

PAM—protospacer adjacent motif;

sgRNA—single guide RNA;

ssDNA—single-strand DNA.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies involving patients or animals as test objects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

All authors equally contributed to experiments design and performing, data analyzing, paper writing and editing.

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