Strategies for Genetic Manipulation of the Halotolerant Black Yeast *H. werneckii*: Ectopic DNA integration and marker-free CRISPR/Cas9 Trasformation

Yainitza Hernandez-Rodriguez[1], A. Makenzie Bullard[2,3], Rebecca J. Busch[4], and José M. Vargas-Muñiz[4,5.]

[1] Department of Biological Sciences, Florida Gulf Coast University, Ft. Myers, FL

[2]Molecular Biology, Microbiology, and Biochemistry Program, School of Biological Science, Southern Illinois University, Carbondale, IL

[3]Microbiology Program, School of Biological Science, Southern Illinois University, Carbondale, IL

[4]Department of Biological Sciences, Virginia Tech, Blacksburg, VA

[5]Early Career Whitman Fellow, Marine Biological Laboratory, Woods Hole, MA

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[#] **Corresponding Author:**

José Vargas-Muñiz
Department of Biological Sciences
Steger Hall
1015 Life Science Cir.
Virginia Tech, Blacksburg, VA 24061
E-mail: j.vargas.muniz@vt.edu

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Abstract

Hortaea werneckii is a halotolerant black yeast commonly found in hypersaline environments. This yeast is also the causative agent of tinea nigra, a superficial mycosis of the palm of the hand and soles of the feet of humans. In addition to their remarkable halotolerance, this black yeast exhibits an unconventional cell division cycle. *H. werneckii* alternates between fission and budding cell division. This cell division cycle is regulated by cell density and the salt concentration in their media. *H. werneckii* have been extensively studied due to their ability to grow in high salinity environments and unconventional cell biology. Nonetheless, deciphering the underlying mechanisms behind these remarkable phenotypes has been limited due to the lack of genetic tools available. Here, we report a new ectopic integration protocol for *H. werneckii* using PEG-$CaCl_2$ mediated protoplast transformation. This approach relies on a selectable marker (hygromycin B) to select for successful integration of the genetic construct. The same construct was used to express cytosolic green fluorescent protein. Finally, we also developed a marker-free CRISPR/Cas9 protocol for targeted gene deletion. These transformation strategies will allow testing hypotheses related to *H. werneckii* cell biology and physiology.

Introduction

Hortaea werneckii (*Dothideales, Ascomycota*) is a black yeast of particular interest due to its ability to grow in high salinity (1). This black yeast is commonly isolated from hypersaline environments, including seawater and solar salterns, and can grow in saturated sodium chloride solution. Not only does this yeast display a remarkable halotolerance, but it also exhibits an unconventional cell division cycle (2). *H. werneckii* first grows in a pattern similar to that of fission yeast. After the first septation, it switches to budding from the poles. This division pattern and cell morphology depend on the growth environment and cell density (3, 4). *H. werneckii* is also of clinical interest due to its ability to cause a superficial mycosis of the hands and feet known as tinea nigra (5–7). Tinea nigra frequently occurs in countries located in the tropics (6). On rare occasions, *H. werneckii* can cause a systemic infection known as disseminated phaeohyphomycosis (7). *H. werneckii* isolates exhibit great phenotypic diversity, including drug susceptibility and pathogenicity variation (3, 8, 9). This phenotypic diversity also correlates with the genetic diversity of *H. werneckii*. *H. werneckii* isolates are intraspecific hybrids of two divergent isolates, contributing to their genetic and phenotypic diversity (10, 11). For these reasons, *H. werneckii* is an emerging model for understanding eukaryotic adaptation to hypersaline environments and how hybridization events contribute to fungal adaptation to extreme environments (9).

Most of our understanding of *H. werneckii* biology and adaptation to hypersaline conditions have been derived from genomics, metabolic, and physiological analyses (1, 9, 18–22, 10–17). However, the lack of reliable genetic tools has limited the ability to

test mechanistic hypotheses related to *H. werneckii* physiology and cell biology. Different methods exist to genetically transform fungi, including protoplast-mediated, *Agrobacterium*-mediate, electroporation, and biolistic (23, 24). These strategies require selectable markers, usually a drug-resistance gene or nutritional gene, to isolate cells that contain the desired construct. Recently, *in vitro* assembled CRISPR/Cas9 transformation systems have been utilized for highly efficient genetic manipulation of fungi (25–30). The CRISPR/Cas9 system has been adapted as a "marker-free" system to perform genome editing without needing a selectable marker (27).

Here, we adapted a protoplast-mediated transformation protocol to integrate a plasmid into the genome of *H. werneckii* ectopically. This ectopic integration strategy can be used for expressing green fluorescent protein (GFP) in the cytoplasm of *H. werneckii.* We also develop a marker-free CRISPR/Cas9 protocol for knocking out genes in *H. werneckii*. These new strategies will further our understanding of the biology of this remarkable yeast.

Materials and Methods

Strains, media, culture conditions

H. werneckii EXF-2000 was use for these studies. Yeast cells were grown in Glucose Minimal Media (GMM [Dextrose 10g/L, Trace Elements, Salt Solution]). Cells were grown at 30˚C for 5 days unless otherwise specified.

Protoplast generation

Hortaea werneckii cells were inoculated in GMM agar plates and incubated at 37˚C for 5-7 days. Cells were harvested and washed once with osmotic media (1.2 M

magnesium sulfate, 10 mM sodium phosphate buffer, pH 5.8). Digest the *H. werneckii* cell wall using osmotic media with 200 mg of Vinotaste for 4-8 hours at 30˚C and shaking at 75 rpm. After digestion, two tubes were combined and overlayed with trapping buffer (0.6M sorbitol, 0.1 M Tris-HCl pH 7). Tubes then were centrifuged for 15 minutes at 3,500 rpm at 4˚C. The cloudy layer (protoplast) at the interface of the osmotic media and trapping buffer was carefully moved into a new 15 mL conical and washed once with ice-cold STC (1.2M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). Protoplasts were resuspended with 1 mL of STC.

PEG-$CaCl_2$-mediated transformation of pUCGH plasmid

200 µL of protoplast was transferred into a 1.5 mL and incubated with 5-15 µg of the pUCGH plasmid for 50 minutes to 1 hour. 1.25 mL of PEG-$CaCl_2$ was added to the protoplast and incubated for 20 minutes. STC was added to protoplast up to 3 mL, and 300 µL were plated to SMM agar plates. Protoplasts were allowed to recover for 24 hours at room temperature. After 24 hours, the protoplast plates were overlayed with SMM top agar with 450 µg/ml of hygromycin B. Colonies appeared between 7-15 days of incubation. Individual colonies were transferred into fresh GMM agar with hygromycin B (150 µg/mL) and incubated at 30˚C.

Imaging EXF-2000

A wet mount of each strain was made by smearing *H. werneckii* cells in 20 µl of distilled water. Cells were then imaged using a widefield microscope (Leica DMi8) using a 100 X oil apochromat objective, and images were captured using a Leica K5 microscope camera.

Assembly of CRISPR/Cas9 RNPs

5 µL of one crRNA, tracrRNA, and nuclease-free duplex buffer (Integrated DNA Technology, IDT) were mixed and heated at 95˚C for 5 minutes. Then, it was cool-down at room temperature to assemble the gRNA. 1.5 µL of each 33 µM gRNA, 1.5 µL of Cas9 nuclease (1µg/µL) (Integrated DNA Technology, IDT), and 22 µl of Cas9 working buffer were mixed and incubated for 5 minutes at room temperature. CRISPR/Cas9 complex was osmotically stabilized by adding an equal volume of 2X STC.

Marker-free CRISPR/Cas9-mediated transformation

Osmotically stabilized CRISPR/Cas9 complex, 70µL of protoplast, and 200 µl of PEG-$CaCl_2$ were gently mixed in a 50 ml conical tube. The protoplast mixture was incubated on ice for at least 30 minutes. 1 ml of PEG-$CaCl_2$ was added and incubated for 15 minutes at room temperature. 325 µl of the protoplast were inoculated into 3 large SMM agar plates and incubated overnight at room temperature. Then, plates were incubated at 30˚C until colonies started appearing on the plates.

Results

Polyethylene glycol (PEG) calcium chloride protoplast-mediated transformation allows for ectopic integration of the pUCGH plasmid.

H. werneckii susceptibility to drugs is determined by the environment (3, 4). We discovered that *H. werneckii* is susceptible to hygromycin B when grown on glucose minimum media, the same media commonly used to grow *Aspergillus fumigatus* (28). Based on this, we decided to use hygromycin B as our selectable marker and used a sorbitol-stabilized glucose minimum media (SMM) to perform our transformations. We

consistently obtained protoplast after 4-8 hours of digesting the cell wall using vinotaste (Novozymes). We then transformed the protoplast using a pUCGH vector, which contains the hygromycin B resistant gene (*hph*) under the control of *Aspergillus nidulans gpdA* promoter and eGFP under the control of the *Aspergillus oryzae* TEF1 promoter (31). We used a high quantity of plasmid to induce ectopic integration (5-15µg) of the plasmid. Protoplasts were allowed to recover for 24 hours before overlaying them with SMM top agar containing hygromycin B (450µg/ml). Hygromycin B-resistant colonies emerged after 7 days of incubation at 30˚C (Fig 1A). Colonies were picked and streaked into a small petri dish containing GMM supplemented with hygromycin B (150µg/ml). Transformants expressed eGFP even after 5 passages in GMM+hygromycin B (Fig 1B). Thus, this approach can be utilized for ectopic integration of markers for cell biology studies.

CRISPR/Cas9 marker-free transformation.

More targeted genetic approaches are needed to further our understanding of *H. werneckii* biology. Similar to other ascomycetes, adapting the PEG-CaCl2 mediated transformation protocol has proven challenging for targeted gene manipulation due to the low homologous recombination rate (32). This challenge is further exacerbated due to the EXF-2000 strain having a significant portion of its genome duplicated due to an intraspecific hybridization event (10). Due to this challenge, we decided to adapt a CRISPR/Cas9 marker-free approach and target the *H. werneckii* melanin synthesis pathway to have a visual phenotype we could screen (27). We targeted a conserved region between *H. werneckii's* two *alb1* alleles: *alb1-1* (BTJ68_00107) and *alb1-2* (BTJ68_01291) using 2 gRNAs (Table 1). Using this approach, approximately 3-5

colonies exhibited an albino phenotype (Figure 2) per plate. These albino colonies were picked and streaked into GMM plates. The albino mutants could still not produce melanin after 5 passages, indicating that we had a stable deletion of the *alb1* alleles. These albino mutants retain the same cell morphology as the wild-type when grown in GMM (Fig 2B), indicating that melanin does not impact cell morphology when grown in GMM Agar.

Discussion

Hortaea werneckii has a unique cell cycle division and can grow at high salt concentrations (3, 4, 33). Due to most isolates being intraspecific hybrids with significant phenotypic variation, *H. werneckii* can potentially become a model for understanding how hybridization leads to novel phenotypes (3, 9). However, the lack of genetic tools has limited the ability to study this organism. Here, we develop two different genetic transformation approaches to further our studies of this unconventional yeast. First, we have developed a plasmid-based ectopic integration protocol. This system can be used to express cellular markers for live cell imaging. For example, we could use it to insert a histone-GFP plasmid and monitor nuclear dynamics. Second, we develop a marker-free CRISPR/Cas9 protocol to target specific genes. One caveat is that our current CRISPR/Cas9 protocol is still largely ineffective and would not be practical for genes that would not have a visual readout. On average, we got 3-5 albino colonies, while other organisms have a 50% to 90% success rate (27–29). However, these organisms were haploid, and only one copy of the gene needed to be mutated, not the 2 copies in *H. werneckii.* It is still possible that we utilized too many protoplasts, and the protoplast

number will need to be titrated to increase efficiency (27). Lastly, as there is a marker system and CRISPR/Ca9 can overcome the homologous recombination rate, we could combine both methods to develop a marker-based CRISPR/Cas9 approach and select those mutants with successful integration of our cassette of interest (28). At the moment of this publication, only one marker has been developed (hygromycin) for *H. werneckii,* but other drugs can be explored to expand the available range of selectable markers.

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Figure Legends

Figure 1. Integration of the pUCGH plasmid in *H. werneckii*. (A) *H. werneckii* EXF-200 is susceptible to hygromycin in GMM media, and hygromycin allows for the selection of ectopic integration of the pUCGH plasmid. Plates were streaked into the respective media, incubated at 37˚C, and photographed after 5 days. **(B)** *H. werneckii*

with integrated pUCGH expresses eGFP under the *A. oryzae* tef1 promoter. Micrographs were obtained using a 100X objective. Scale bar, 20 µm.

Figure 2. Generation of albino mutants using CRISPR/Cas9. (A) The *alb1*Δ/Δ fails to melanize after prolonged incubation in GMM. Plates were streaked and incubated at 30˚C for 5 days before being photographed. **(B)** *alb1*Δ/Δ have similar morphology as the parent EXF-2000 strain when grown in GMM agar. Micrographs were obtained using a 100X objective. Scale bar, 20 µm.

Table 1: crRNAs used in this study

crRNA	**Target**	**Sequence**
alb1-gRNA-5-1	5' of *alb1*	AAGCGCUUGACGGUGGUUGGCGG
alb1-gRNA-5-2	*5' of alb1*	AGCGUUGCGCGAAAGCGCUGCGG

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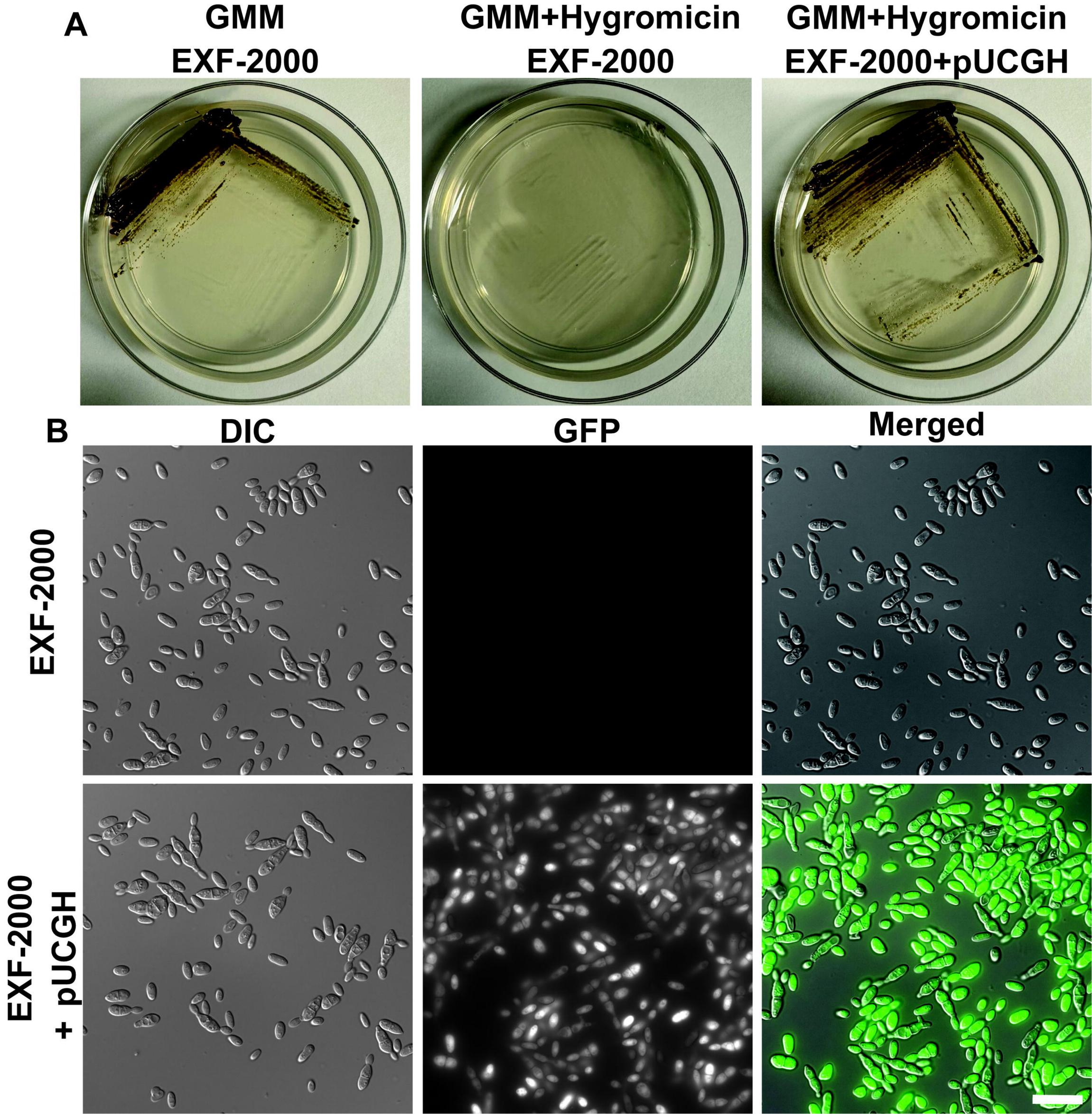
Figure 1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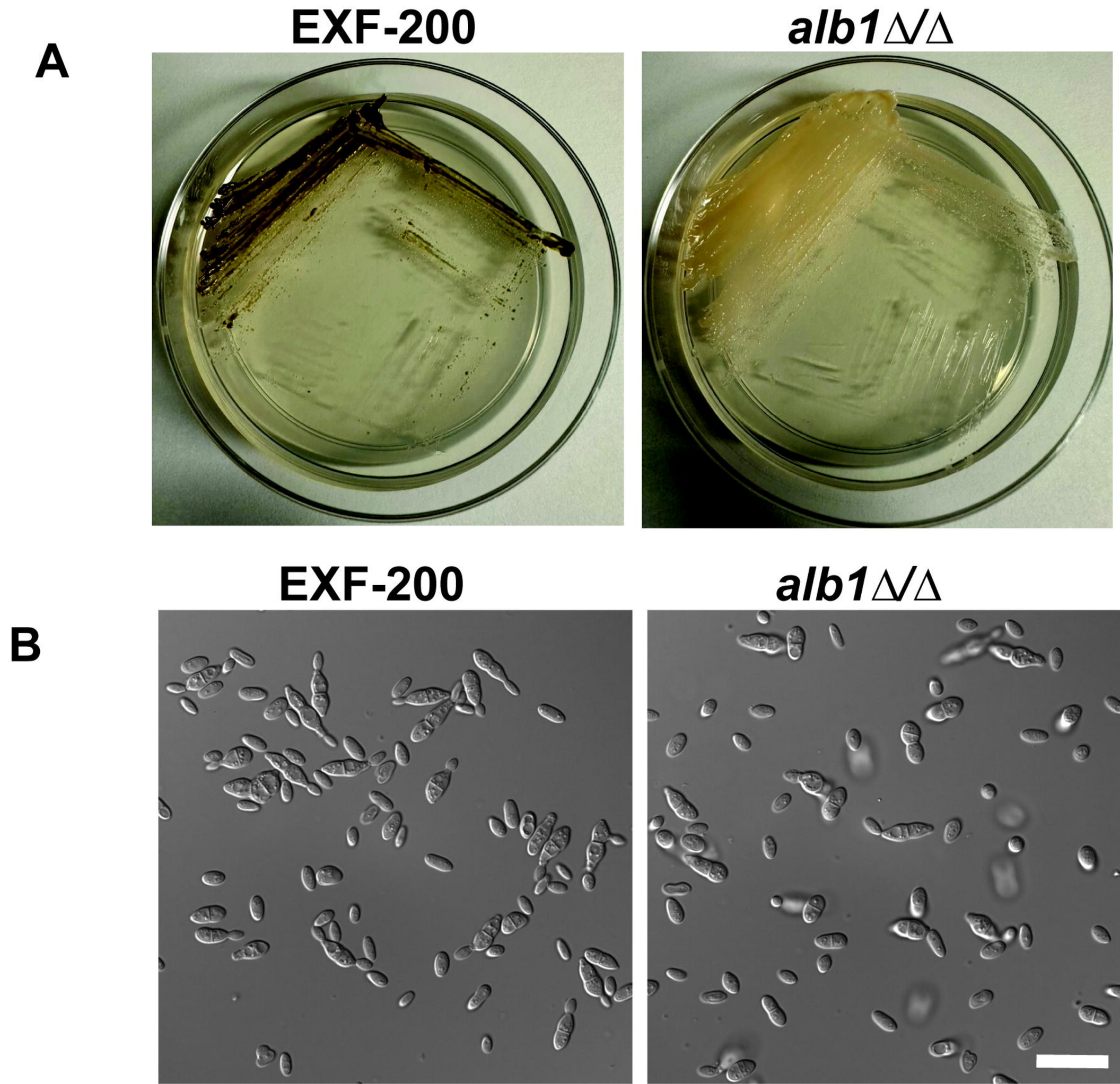
Figure 2