A Modular Cloning Toolkit Including CRISPRi for the Engineering of the Human Fungal Pathogen and Biotechnology Host Candida glabrata

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Technical Note

A Modular Cloning Toolkit Including CRISPRi for the Engineering of the Human Fungal Pathogen and Biotechnology Host *Candida glabrata*

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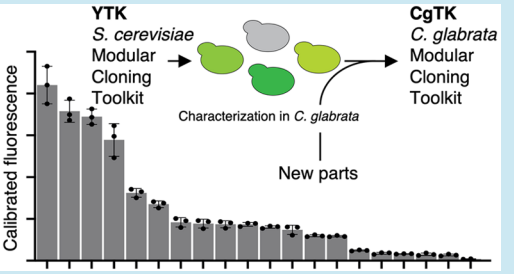
ABSTRACT: The yeast *Candida glabrata* is an emerging, often drug-resistant opportunistic human pathogen that can cause severe systemic infections in immunocompromised individuals. At the same time, it is a valuable biotechnology host that naturally accumulates high levels of pyruvate—a valuable chemical precursor. Tools for the facile engineering of this yeast could greatly accelerate studies on its pathogenicity and its optimization for biotechnology. While a few tools for plasmid-based expression and genome engineering have been developed, there is no well-characterized cloning toolkit that would allow the modular assembly of pathways or genetic circuits. Here, by characterizing the *Saccharomyces cerevisiae*-based yeast molecular cloning toolkit (YTK) in *C. glabrata* and by adding missing components, we build a well-characterized CgTK (*C. glabrata* toolkit). We used the CgTK to build a CRISPR interference system for *C. glabrata* that can be used to generate selectable phenotypes via single-gRNA targeting such as is required for genome-wide library screens.



KEYWORDS: *Candida glabrata, modular cloning toolkit, Golden Gate cloning, CRISPR interference*

INTRODUCTION

Candida glabrata is an opportunistic human fungal pathogen, causing 10−25% of fungal bloodstream infections in humans.[1−5] Clinical isolates are increasingly resistant to multiple drugs, and as a consequence, mortality rates of blood-stream infected individuals are high.[6−9] Unlike other fungal pathogens that are acquired from the environment, *C. glabrata* is a natural commensal of the human mycobiome, mostly the GI tract,[1,10,11] but it can overgrow and turn virulent once the immune system of the human host is compromised. The virulent phenotype is linked to *C. glabrata*'s ability to grow rapidly at 37 °C, its high capacity for adhesion and biofilm formation, its intrinsic ability to tolerate certain antifungal drugs, and its rapid adaptation to stresses.[8,12−14] Although much progress has been made in understanding *C. glabrata*'s biology, the molecular regulatory underpinnings that enable the phenotypic adaptation to a pathogenic lifestyle are not yet understood.

At the same time, some *C. glabrata* strains are used as valuable chassis for biotechnology as they naturally produce high levels of pyruvic acid[15,16]—an important precursor for agrochemistry and a dietary supplement—and they have been engineered to metabolize pyruvic acid into valuable downstream products such as α-ketoglutarate,[17] fumarate,[18] acetoin,[19] malate,[20] and diacetyl.[21]

Tools enabling a synthetic biology approach to *C. glabrata*'s biology could not only facilitate its metabolic engineering toward biotechnological applications but also help elucidate the genetic and regulatory architectures underlying the phenotypic transition to virulence.[22] In addition, understanding the genetic design of this transition could become a valuable inspiration for Synthetic Biology and help design sophisticated programmed cellular behavior.

Some genetic tools exist for *C. glabrata*, such as a set of expression plasmids,[23] several nonhomologous-end-joining-based CRISPR tools,[24,25] as well as transposons for gene disruption[26,27] and a deletion collection covering 12% of the *C. glabrata* genome.[28] Here, we extend the genetic toolbox for *C. glabrata* by building a molecular cloning toolkit (CgTK) by recharacterizing and extending the Yeast Toolkit (YTK) built for *Saccharomyces cerevisiae*. Modular cloning kits based on Golden Gate assembly[29] have proven indispensable to overcome cloning barriers for bacterial and eukaryotic hosts, including plants.[30−33]

RESULTS AND DISCUSSION

Design of the *Candida glabrata* Toolkit (CgTK). Given the close relationship between *S. cerevisiae* and *C. glabrata*,[12]

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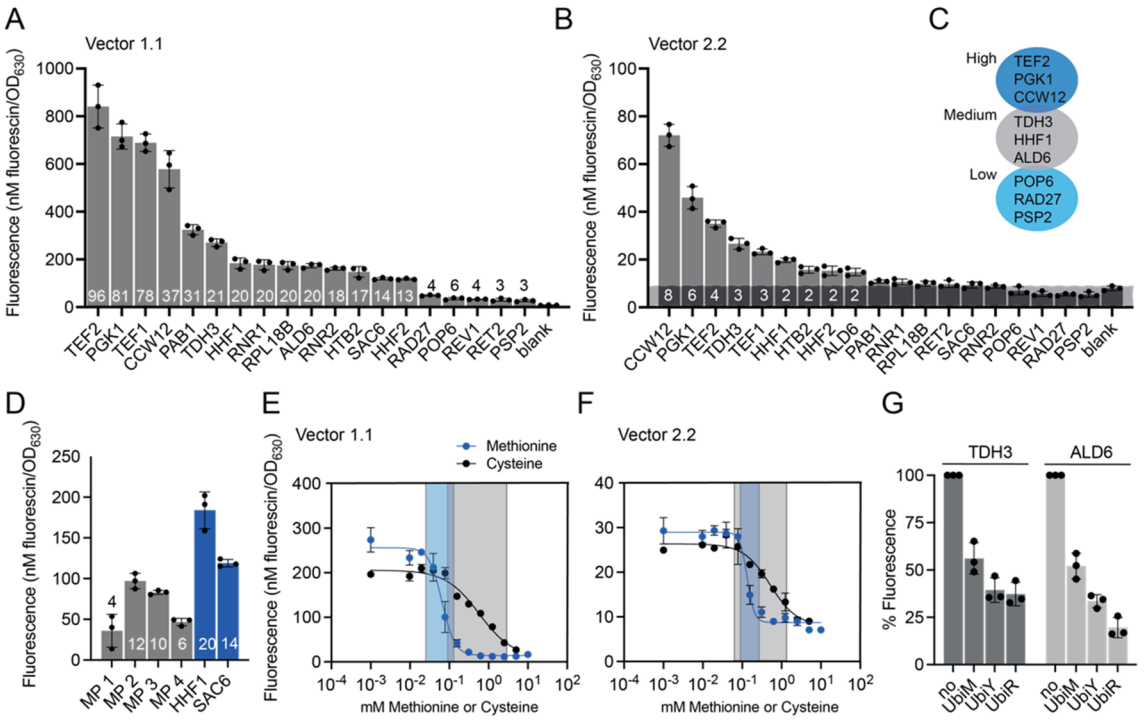

Figure 1. Performance of YTK and additional parts in *C. glabrata* using green fluorescence (Venus) as readout. (A and B) Performance of the 19 YTK promoters in *C. glabrata* using two different vectors: version 1.1 (A) and 2.2 (B) and using the YTK-derives Venus and the ENO1 terminator as readout. The numbers indicate the fold-change in fluorescence over background, where background is the autofluorescence of *C. glabrata* cells. Measurements were taken after 16 h of growth (see Materials and Methods in the SI). Note: The *Y*-axis shows a different scale. Units were calibrated as described in the Materials and Methods. (C) Suggested high, medium, and low expression promoters based on the data displayed in A and B. (D) Performance of minimal yeast promoters in *C. glabrata* using vector version 1.1, promoters are compared to the YTK-derived HHF1p and SAC6p. (E and F) Performance of MET3 promoters in vector version 1.1 (E) and 2.2 (F) when grown in the presence of decreasing concentrations of methionine (starting from 10 mM methionine and 5 mM cysteine, 2-fold dilutions were measured). Note: 10 mM cysteine led to partial growth inhibition (Supplementary Figure S11); this is why values starting from 5 mM are displayed. The gray and blue shaded boxes indicate the operational range of the promoter repressed methionine (blue) and repressed with cysteine (gray). (G) Performance of the protein degradation tags Ubi-M, Ubi-Y, and Ubi-R in *C. glabrata* using vector version 1.1. All experiments were run in biological triplicates (three transformants), and error bars represent the standard deviation. For all experiments, fluorescence measurements were taken after 16 h of growth (see Materials and Methods).

we reasoned that the most resource-saving way to build the CgTK was to use the *S. cerevisiae* YTK and characterize part performance in *C. glabrata*, identify malperforming parts, and add missing ones. In total, we characterized 21 constitutive promoters (19 YTK, 2 new), four constitutive synthetic minimal promoters (4 new), three inducible promoters (1 YTK, 2 new), and three protein degradation tags (3 YTK). We constructed eight vector sets for the assembly of (multiple) transcriptional units featuring four auxotrophic markers and two origins of replication and we built a CRISPRi system (Supplementary Figure S1 and Supplementary Tables S1 and S2). We used *C. glabrata* ATCC 2001 HTL^{-}[28] as a host, a clinical isolate frequently used for research including for the creation of the *C. glabrata* deletion collection.[28]

A Set of Cloning Vectors. First, we created an eight-membered green-white screening compatible vector set featuring four selection markers combined with either the YTK-derived ScCEN/ARS origin of replication from *S. cerevisiae* or a *C. glabrata* derived CgCEN/ARS. Most *C. glabrata* research relies on plasmids encoding the CgCEN/ARS, while most available yeast plasmids (e.g., on Addgene) contain an ScCEN/ARS. To facilitate the use of plasmids designed for *S. cerevisiae* in *C. glabrata*, we were interested in whether the ScCEN/ARS sequence is functional in *C. glabrata*.

We recorded growth curves to detect potential plasmid burden using "empty" vectors and vectors carrying an HHF1p-Venus-ENO1t transcriptional unit (Supplementary Figures S2 and S3). Vector-specific plasmid burden had been shown before for *S. cerevisiae* and can help in choosing the right vector for an application.[34] Cells carrying the empty vectors showed overall similar growth behavior on their respective selective media when compared to cells not carrying a vector on nonselective media (Supplementary Figures S2B and C). Only vector version 1.3 caused slightly slowed growth and reduced final OD_{630} (Supplementary Figure S2B). The impact of the *LEU2* marker on growth in *C. glabrata* is consistent with results in *S. cerevisiae*.[34] In contrast, cells carrying the vector set encoding a transcriptional unit and the ScCEN/ARS showed pronounced growth phenotypes with longer lag phases and lower final OD_{630} (Supplementary Figure S3, Note S1).

Copy Number Differences Across the Vector Set Allow for Tuning Expression Strength. Given the growth phenotypes, we tested for vector-dependent differences in expression levels and in plasmid copy number: The ScCEN/ARS-based vectors showed a 3- to 8-fold higher expression level when compared to their CgCEN/ARS-based counterparts (Supplementary Figure S4). Relative copy numbers of some of the vectors were compared by qPCR (Supplementary Figure S5) and showed that vector version 1.1 was the most abundant

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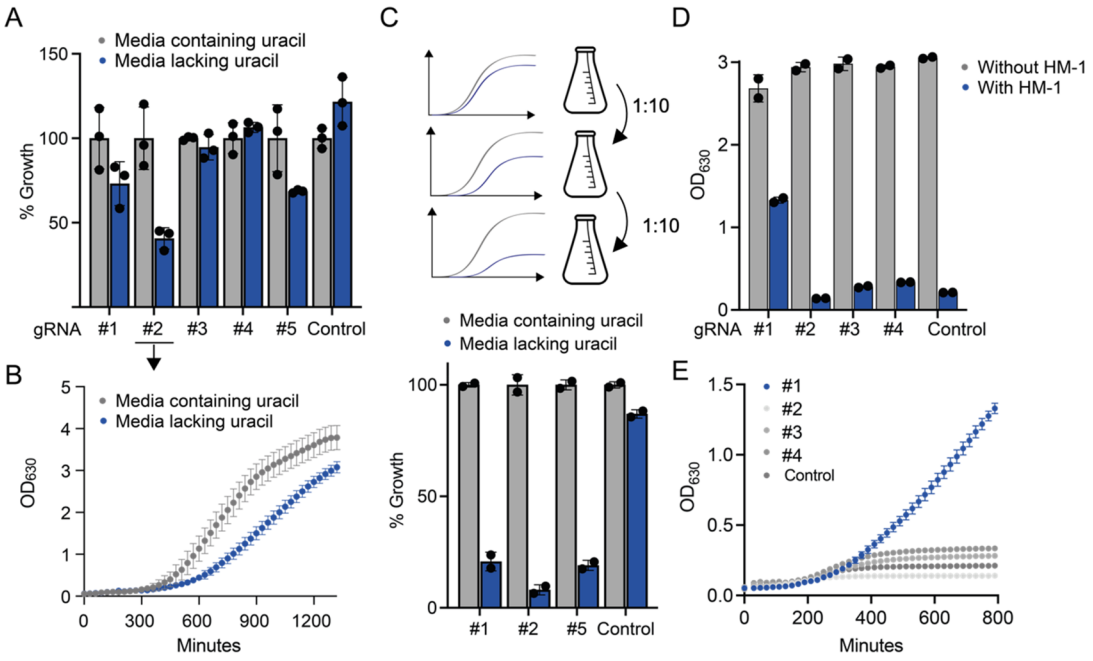

Figure 2. CRISPRi in *C. glabrata*. (A) Five gRNAs were tested for repression of the *URA3* gene by measuring growth in media lacking uracil. Percent growth (OD_{630}) after 12 h when compared to the same strain grown in media with uracil is depicted. The control uses a gRNA that does not target the *URA3* promoter. (B) Full growth curve of the strain harboring the best performing gRNA #2 in media containing uracil and media lacking uracil. (C) Depletion of strains harboring gRNAs #1, #2, and #5 after three serial dilutions in media lacking uracil when compared to growth in media containing uracil. Strains were grown in the presence or absence of uracil in duplicate, and cultures were diluted 1:10 into fresh media after 7 to 10 h. Final OD_{630} after the third dilution round is given as percentage of growth in media containing uracil. (D) Four gRNAs were tested for repression of the *ALG3* gene by measuring growth in media containing the killer toxin HM-1. The control uses a gRNA that does not target the *ALG3* promoter. (E) Growth curves of the strains harboring the targeting or nontargeting RNAs in media containing HM-1.

vector, while vector version 2.2 was the least abundant (Supplementary Figure S4A). Our data indicate that not only the origin of replication but also the selection marker seems to impact copy number. Further, copy number alone does not seem to be responsible for growth burden or expression levels: For example, vector 2.1 seems to be as abundant as vector 1.2, but it shows less growth burden (Supplementary Figure S3) but also lower expression (Supplementary Figure S4). These findings are in line with plasmid-growth-burden-related findings for *S. cerevisiae*, supporting the need for good experimental characterization of parts to allow predictable engineering.[34]

Twenty-One Constitutive Yeast Promoters and Four Synthetic Minimal Promoters. Next, we characterized all 19 YTK-derived constitutive promoters in *C. glabrata* using the highest expression vector 1.1 and one of the low expression vectors, vector version 2.2. All 19 YTK promoters were functional in *C. glabrata*, showing at least 3-fold expression over background in vector version 1.1 and covering 2 orders of magnitude in expression strength (Figure 1A). Expression was lower in vector version 2.2: 8- to 30-fold for the six strongest promoters (Figure 1B, Supplementary Figure S6). The exact order of promoter strength slightly varied between vectors, but a set of strong, medium, and weak promoters could be identified (Figure 1C). Several of the promoters showed different strength profiles in *C. glabrata* when compared to *S. cerevisiae* (Supplementary Figure S7) and the highest expression within the set of promoters was 2.7-fold lower in *C. glabrata* when compared to *S. cerevisiae* (Supplementary Figure S8B). To potentially reach higher expression in *C. glabrata* we tested two *C. glabrata*-derived central carbon metabolism promoters (Supplementary Figure S9). Neither showed higher expression than the YTK-promoters, but still medium to strong expression (27- to 49-fold over background). We tested a subset of seven promoters with a red fluorescent protein (mRuby2) as readout and obtained similar results (Supplementary Figure S10, Note S2).

We further tested four constitutive minimal synthetic promoters (MPs) originally engineered for *S. cerevisiae*,[35] herein called MP1 to MP4 (Note S3). All four promoters were functional in *C. glabrata* yielding between 4- and 12-fold expression over background (Figure 1D). None of the MPs were strong, but they could be useful as short, low to medium expression promoters in *C. glabrata* and could likely be further optimized (Note S3).

Three Inducible Promoters. As the YTK-derived galactose promoter is not functional in *C. glabrata*, we characterized two inducible *C. glabrata* promoters: the methionine and/or cysteine repressible promoter MET3p and the *C. glabrata* copper(II) sulfate ($CuSO_4$)-inducible promoter MT-1p. In addition, we tested the YTK-derived $CuSO_4$-inducible CUP1 promoter. The MET3 promoter in vector 1.1 allowed for a 17-fold change in Venus expression when using methionine as a repressor and promoter leakiness was 2-fold over background (Figure 1E and Supplementary Figure S12A). When using cysteine as a repressor, a 7-fold change in expression was achieved. The reduction in fold change was due to cysteine being a less effective repressor. At full cysteine repression (≥5 mM cysteine), leakiness was still 3-fold over background. However, using cysteine as a repressor widened the operational range of the promoter when compared to methionine (Figure 1E). Similar results, but lower expression levels were achieved when using vector version 2.2 (Figure 1F and Supplementary Figure S12B). Also,

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the two $CuSO_4$-inducible promoters CUP 1p and MT-1p were functional in *C. glabrata* (Supplementary Figure S14, Note S4).

Ubiquitin-Based Degradation Tags. N-terminal degradation tags (N-degrons) of different strength allow for tuning protein levels by protein turnover rates,[36] and have proven to be useful tools for functional proteomics[37] and genomics,[38] fine-tuning of genetic circuit behavior,[39] or temporal control over gene functions.[38] Here we show that the ubiquitin-based YTK-derived degradation tags can be functionally ported into *C. glabrata* (Figure 1G, Supplementary Figure S15, and Note S5).

A CRISPRi System for *C. glabrata*. Finally, we used the CgTK to build a CRISPRi system based on the deactivated Cas9 (dCas9) fused to the transcriptional repression domain Mxi1,[40] in combination with the gRNA design featured in the YTK (Note S6). We chose to test repression of genes by a single gRNA as we are most interested in using the CRISPRi system for functional genomics studies where screens are based on the fact that repression by single gRNAs generates selectable phenotypes such that cells carrying a given gRNA can be enriched or depleted over growth and dilution cycles.

We used the *URA3* gene (CAGL0I03080g) and the *ALG3* gene (CAGL0A04587g) to show the functionality of our CRISPRi system: The repression of *URA3* should lead to a selectable phenotype on media lacking uracil. *ALG3* is a functional yet unverified gene in *C. glabrata*, but its *S. cerevisiae* orthologue encodes for an alpha-1,3-mannosyltransferase involved in (membrane)-protein glycosylation. An *ALG3* deletion renders *S. cerevisiae* resistant to the yeast killer toxin HM-1. As we are interested in using the CRISPRi system to study resistant phenotypes to yeast killer toxins, we verified that an *ALG3* deletion would also render *C. glabrata* resistant to this toxin (Supplementary Figure S16) and subsequently aimed to verify that its repression by CRISPRi also changes the resistance profile. We designed four to five gRNAs targeting the promoters of *URA3* and *ALG3* in a window of −50 to +300 bp respective to the transcriptional start site (TSS) (Supplementary Table S5)[41] based on previous design rules in *S. cerevisiae*[42,43] and mammalian cells. First, we tested the *URA3*p targeting CRISPRi system by measuring growth in media with and without uracil. For three out of the five gRNAs, we observed a slow growth phenotype in the absence of uracil (Figure 2A and B), which allowed to deplete the gRNA-encoding strains over three growth-and-dilution cycles to 4 to 10-fold (depending on gRNA) when compared to the same strain grown in the presence of uracil or a strain not expressing a targeting gRNA (Figure 2C). Next, we assessed the performance of the *ALG3*p targeting CRISPRi system: Here one out of the four tested gRNAs could be enriched in the presence of the killer toxin HM-1 (Figure 2D and E).

CONCLUSION

Here we deliver a molecular cloning kit for the human fungal pathogen and biotechnology host *C. glabrata* by characterizing the performance of the YTK and by adding several new parts.

The strongest promoters generated expression levels 100-fold over background and allow a user to tune expression levels over 2 orders of magnitude. The herein characterized vector-set allows for further tuning expression strength as they have different copy number. To further enhance the expression strength of the promoters, it might be possible to choose better suited "seams" for the Golden Gate assembly of type 2 (promoter) and type 3 (open reading frame) parts as the YTK-inherent *Bgl*II site was shown to be suboptimal.[44]

The presented single-gRNA CRISPRi system is useful for library selections and could likely be extended to generate complete knock-down phenotypes (as required for metabolic engineering) by using several gRNAs that target the same promoter simultaneously. YTK-compatible multi-gRNA cloning strategies are readily available to extend the herein presented single-gRNA system.[45]

In summary, we think that the CgTK is a starting point for effective metabolic engineering and phenotypic characterization of *C. glabrata*.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00560.

Materials and Methods; Supplementary Tables S1−S5; Supplementary Figures S1−S17; Notes S1−S6 (PDF)

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Author Contributions

SB designed the work, performed experiments, and wrote the paper. RCP cloned and tested the BsaI- and BsmBI-site free dCas9-MxiI. MM performed qPCR experiments.

Notes

The authors declare no competing financial interest.

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