



Development of genetic markers in *Yarrowia lipolytica*

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Abstract

The oleaginous yeast *Yarrowia lipolytica* represents a potential microbial cell factory for the recombinant production of various valuable products. Currently, the commonly used selection markers for transformation in *Y. lipolytica* are limited, and successive genetic manipulations are often restricted by the number of available selection markers. In our study, we developed a dominant marker, *dsdA*, which encodes a D-serine deaminase for genetic manipulation in *Y. lipolytica*. In *Y. lipolytica*, this marker confers the ability to use D-serine as a nitrogen source. In addition, the selection conditions of several infrequently used dominant markers including *bleoR* (zeocin resistance), *kanMX* (G418 resistance), and *guaB* (mycophenolic acid resistance) were also analyzed. Our results demonstrated that these selection markers can be used for the genetic manipulation of *Y. lipolytica* and their selection conditions were different for various strains. Ultimately, the selection markers tested here will be useful to expand the genetic toolbox of *Y. lipolytica*.

Key points

- The *dsdA* from *Escherichia coli* was developed as a dominant marker.
- The applicability of several resistance markers in *Y. lipolytica* was determined.
- We introduced the *Cre*/mutant *lox* system for marker recycling.

Keywords *Yarrowia lipolytica* · Marker removal · Dominant marker · *Cre/lox*

Introduction

The non-conventional yeast *Yarrowia lipolytica* is an attractive microbial cell factory for the recombinant production of various chemicals. Due to its unique physiological characteristics including high tricarboxylic acid cycle (TCA) flux and abundant acetyl-CoA supply, it has been used to produce many valuable chemicals such as organic acids, lipids, terpenoids, polyketides, and flavonoids (Fickers et al. 2020; Chen et al. 2021; Zhang et al. 2022; Yu et al. 2018; Lv et al. 2019).

Engineering cell factories often requires genetic markers to select the cells with desired genetic modifications. Successive genetic manipulations are often limited by the number of available selection markers. The CRISPR/Cas system has been developed for genomic engineering in *Y. lipolytica*; however, *Y. lipolytica* has a preference for

non-homologous end joining (NHEJ) and manipulation of key NHEJ or homologous recombination proteins is necessary to improve the efficiency of targeted genome integration (Schwartz et al. 2016; Wu et al. 2023). In contrast, marker-based random integration through NHEJ can be easily achieved. We previously demonstrated that random integration can be used to construct a gene expression library for high producer selection (Cui et al. 2019; Liu et al. 2022). Therefore, in metabolic engineering, genetic markers are required for gene manipulation. Currently, the most commonly used selectable markers in *Y. lipolytica* can be divided into auxotrophic and dominant markers (Larroude et al. 2018). The most common auxotrophic markers mainly include *LEU2*, *URA3*, and *TRP1* (Barth and Gaillardin 1996), while the most frequently used dominant markers include *hphMX* (hygromycin resistance) and *NAT1* (nourseothricin resistance) (Cordero Otero and Gaillardin 1996; Kretzschmar et al. 2013). In addition, *bleoR* (zeocin resistance), *guaB* (mycophenolic acid resistance), and *CRF1* (Cu²⁺ resistance) were reported as dominant markers in *Y. lipolytica* (Tsakraklides et al. 2018; Wagner et al. 2018; Wang et al. 2011). Moreover, Hamilton et al.

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(2020) showed that *YIAMDI* (encoding acetamidase) could be selected on acetamide media and counter-selected on fluoroacetamide media, while Vandermies et al. (2017) reported that *EYK1* (encoding erythrulose kinase) can be used as an efficient catabolic selectable marker for genome editing. Loss of the *MET25* gene (encoding *O*-acetyl homoserine sulfhydrylase) induces lead sulfide (PbS) aggregation in media supplemented with divalent lead, such that deficient cells become brown/black, providing a positive/negative color-associated selection marker in *Y. lipolytica* (Edwards et al. 2020). Furthermore, Larroude et al. (2020) did a phenotype screen by disrupting *GSY1*, *MFE1*, or *LIP2* lipid metabolism genes to test CRISPR/Cas9 editing success rate. Despite these useful markers, other dominant selection markers are being sought to expand the genetic engineering toolbox for this species.

Marker recycling is necessary for iterative genetic engineering. The Cre/*loxP* system from bacteriophage P1 is a frequently used site-specific recombination system (Sauer 1987). The *loxP* site is a 34 bp sequence composed of two 13-bp inverted repeats separated by an asymmetric 8-bp core sequence (Hoess et al. 1982). When two *loxP* sites in the same orientation flank a marker gene, the expression of Cre recombinase will result in excision of the marker gene between the two *loxP* sites. Fickers et al. (2003) established the Cre/*loxP* system for marker recycling in *Y. lipolytica*. Using this system to rescue markers is highly efficient; however, recombinant *loxP* copies left in the genome may interfere with subsequent rounds of recombination (Suzuki et al. 2005a, b). This problem can be avoided by using mutant *loxP* sites. One class of mutated *loxP* site is applied in the left element/right element (LE/RE) mutant strategy. The Cre recombinase can mediate recombination between the LE mutant *loxP* site and the RE mutant *loxP* site, generating a double-mutant *loxP* site that remains in the genome and a wild-type *loxP* site in the excised fragment. After marker recycling, the double-mutant *loxP* site in the genome is poorly recognized by Cre recombinase, which prevents further genome rearrangement (Albert et al. 1995). In addition to the Cre/*loxP* system, Wagner et al. (2018) developed the piggyBac transposon system to achieve footprint-free marker recycling.

In this study, we developed *dsdA* from *Escherichia coli*, which encodes a D-serine deaminase as a dominant marker. In *Y. lipolytica*, it also confers the capability to use D-serine as the sole nitrogen source. In addition, we also determined the selection conditions of different dominant markers including *kanMX*, *bleoR*, and *guaB* (Table 1). The markers documented here expand the available genetic tools for *Y. lipolytica* and may facilitate the engineering of *Y. lipolytica* strains to produce various chemicals.

Materials and methods

Strains and media

The yeast strains used were *Y. lipolytica* PO1f (ATCC MYA-2613, *matA leu2-270 ura3-302 xpr2-322 axp-2*), W29 (ATCC 20460, *CLIB89 matA* wild type), Y1444, and Y31248 (from the China Center of Industrial Culture Collection, CICC). A YPD medium (10 g/L yeast extract, 20 g/L tryptone, and 20 g/L glucose) was used to cultivate yeast cells under non-selective conditions. The medium that consisted of SC-URA is 1.7 g/L YNB (Yeast Nitrogen Base without amino acids and ammonium sulfate) (BBI Life Science Corporation, Shanghai, China) (see Supplemental Table S3 for the ingredients of YNB), 5 g/L (NH₄)₂SO₄, and 0.77 g/L CSM-URA (Sunrise Science Products, Knoxville, USA) (see Supplemental Table S4 for the ingredients of CSM-URA). The DH5 α (TransGen Biotech Co., LTD, Beijing, China) of *E. coli* was used for plasmid construction and propagation. *E. coli* was cultivated in a Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with 0.1 to 0.2 mg/mL ampicillin at 37 °C (Liu et al. 2021). For solid media, 20 g/L of agar was added to the media.

For drug testing, the *Y. lipolytica* strains were revived in YPD medium at 30 °C with shaking at 200 rpm for 12 h. After overnight incubation, the cells were plated on YPD agar plates with different concentrations of antibiotics at 30 °C for 3 days. G418 was added at the concentrations of 1 mg/mL, 2 mg/mL, 4 mg/mL, and 7.2 mg/mL, respectively. Zeocin was added at the concentrations of 0.2 mg/mL, 0.4

Table 1 Summary of the markers used in our study and the selection conditions for the PO1f strain

| Genetic markers | Chemicals | Reactions | Concentration | Medium |
|-----------------|-------------------|---|---------------|---|
| <i>dsdA</i> | D-Serine | D-Serine deaminase converts D-serine to pyruvate and ammonia | 0.5 mg/mL | YNB without (NH ₄) ₂ SO ₄ + glucose |
| <i>kanMX</i> | G418 | Aminoglycoside 3-phospho-transferase | 7.2 mg/mL | YPD |
| <i>bleoR</i> | Zeocin | Encoding a small acidic protein (MW 13665) that binds to zeocin | 0.8 mg/mL | YPD |
| <i>guaB</i> | Mycophenolic acid | Inosine monophosphate dehydrogenase | 0.5 mg/mL | YPD |

mg/mL, 0.6 mg/mL, and 0.8 mg/mL, respectively. Mycophenolic acid was added at the concentrations of 0.1 mg/mL, 0.3 mg/mL, and 0.5 mg/mL, respectively. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added at the concentrations of 1.6 mg/mL, 3.2 mg/mL, 4.39 mg/mL, 4.55 mg/mL, 4.7 mg/mL, 5 mg/mL, and 6.3 mg/mL, respectively. The lowest concentration without any colony arising (minimal inhibition concentration) was taken as the required level of antibiotics to select transformants. The expression of *dsdA* was chosen in a D-serine selection medium containing 20 g/L glucose and 1.7 g/L YNB. Concentrations of 100 $\mu\text{g/mL}$, 125 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$, and 500 $\mu\text{g/mL}$ D-serine were added, respectively. Uracil and leucine were added if necessary.

Plasmid construction and strain construction

The *dsdA* (from *E. coli*), *kanMX* (from bacteria), *bleoR* (from *Streptoalloteichus hindustanus*), and *guaB* (from *E. coli*) genes were codon-optimized (Supplemental Material) (GenScript Co., LTD, Nanjing, China). The *CRF1* gene was PCR amplified from W29 genomic DNA. The marker genes were assembled under the control of the *EXP1* promoter and the *ICL1* terminator with the YLEP-ET2 plasmid (Cui et al. 2019) to generate the episomal plasmids YLEP-ET2-*dsdA*, YLEP-ET2-*kanMX*, YLEP-ET2-*bleoR*, YLEP-ET2-*guaB*, and YLEP-ET2-*CRF1* (Supplemental Fig. S1). These plasmids were transformed into the *E. coli* strain DH5 α for propagation. Finally, the plasmids were transformed into the yeast strain using a lithium acetate method (Chen et al. 1997).

Genetic marker testing

The plasmids with a resistance gene or linear DNA fragments (obtained by PCR) containing a resistance gene were transformed into the yeast, and cells were plated onto YPD agar supplemented with working concentrations of an antibiotic. After incubation for 3 days at 30 °C, the genomic DNA of transformants was extracted to amplify the target gene. The resulting PCR products were then sequenced to confirm the presence of a marker gene. The primers used in this study are listed in Supplemental Table S1. Furthermore, real-time quantitative reverse transcription PCR (RT-qPCR) was used to confirm the expression of *dsdA*, *kanMX*, *guaB*, and *bleoR*. For the *dsdA* selection, the nitrogen source was replaced with D-serine in the selection medium.

RT-qPCR

For RT-qPCR, the cells with a marker gene were revived in a YPD liquid medium supplemented with the working concentrations of an antibiotic (30 °C, 200 rpm for 8 to 12 h). The RNA was extracted and purified using a UNIQ-10 column

TRIzol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China). Next, cDNA was synthesized with the PrimeScript RT-PCR Kit (TaKaRa, Shiga, Japan). RT-qPCR was performed by using an Applied Biosystems Quant-Studio 3 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) with the SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan). The *ACT1* gene (encoding yeast actin, GenBank accession number YALI0_D08272g) was used as the reference gene to quantify the relative expression levels of marker genes.

Confirmation of the *lox66-URA3-lox71* cassette

The *EXP1p-URA3-ICL1t* fragment was amplified from the YLEP-ET2-URA3 plasmid, and the *lox66* and *lox71* sequences (Suzuki et al. 2005a) were synthesized by Tsingke Biotechnology Co., Ltd (Qingdao, China). The two mutant *lox* sequences were inserted on the alternate sides of the *EXP1p-URA3-ICL1t* fragment, and the resulting fragment was named the *lox66-URA3-lox71* cassette. The *lox66-URA3-lox71* cassette was then transformed into PO1f (PO1f-URA3), and SC-URA plates were used to screen positive transformants. The cassette was further verified by PCR and sequencing. Next, the YLEP-Leu-Cre plasmid (Cui et al. 2017) was transformed into the positive transformants to produce Cre recombinase. After transformation, the colonies were picked and incubated on SC-URA and YPD agar plates, respectively, to confirm the deletion of the *URA3* marker.

Nucleotide sequence accession numbers

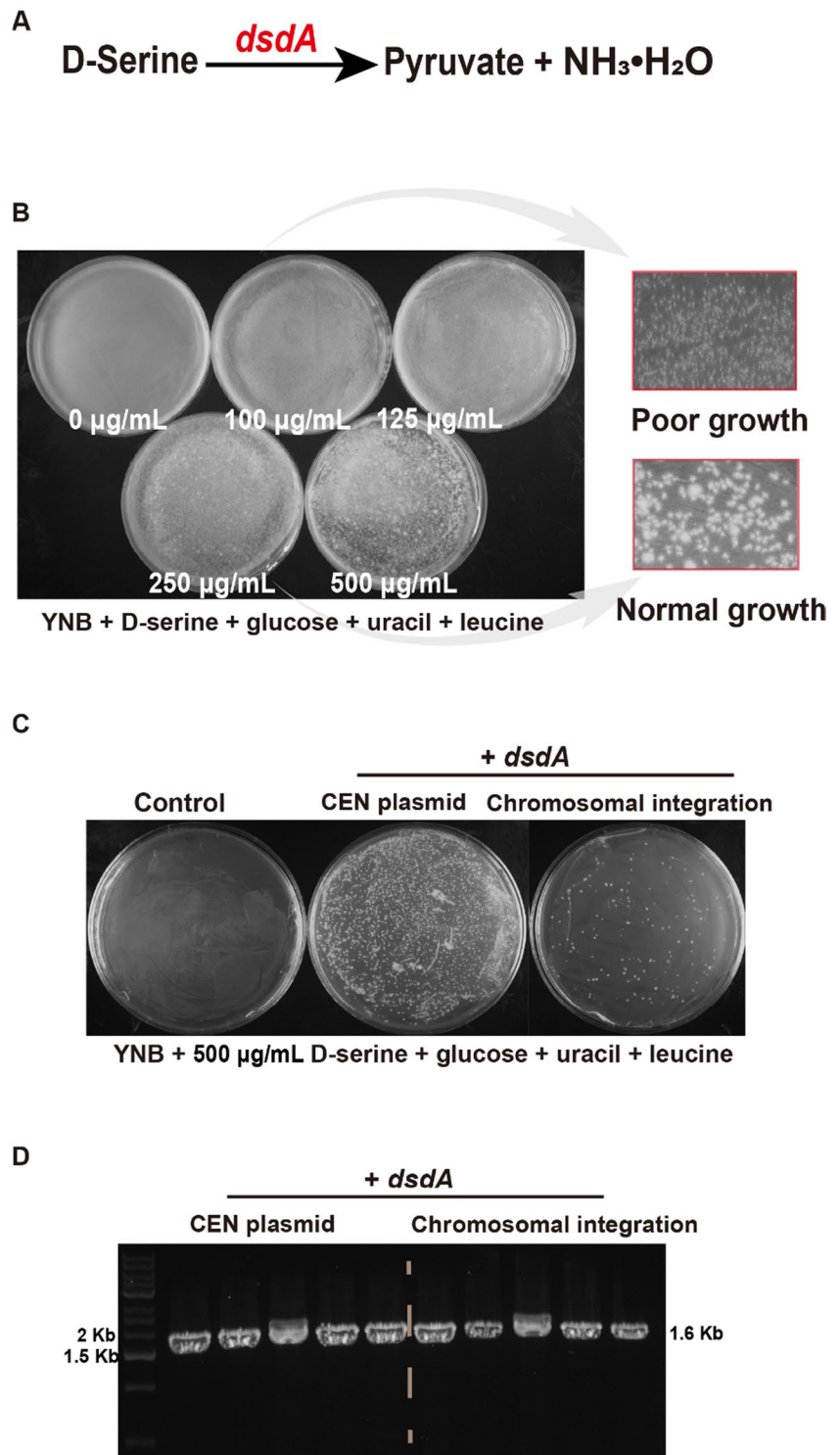
The nucleotide sequences of *dsdA*, *kanMX*, *bleoR*, and *guaB* were deposited in the NCBI Sequence Read Archive under the BioProject PRJNA961308. The *CRF1* GenBank accession number of *Y. lipolytica* is XM_500631.1. Their sequences are also provided at the end of the Supplemental Material.

Results

Identification of *dsdA* as a selectable marker in *Y. lipolytica*

It is reported that the *dsdA* gene from *E. coli* encodes a D-serine deaminase that converts D-serine to pyruvate and ammonia, which confers host resistance to D-serine and the ability to use D-serine as the nitrogen source (Cosloy and McFall 1973; Maas et al. 1995) (Fig. 1A). To determine whether *dsdA* can work in *Y. lipolytica*, we first tested whether *Y. lipolytica* can grow in defined medium containing D-serine as the sole nitrogen source. We found that PO1f

Fig. 1 *dsdA* confers host resistance to D-serine and the ability to use D-serine as a nitrogen source in the *Y. lipolytica* strain PO1f. **A** *dsdA* encodes a D-serine deaminase that converts D-serine to pyruvate and ammonia. **B** Determining the concentration of D-serine to maintain the growth of PO1f after the transformation of *dsdA*. **C** The growth of the *dsdA* expression strains on 500 $\mu\text{g/mL}$ D-serine. The transformants were plated on 500 $\mu\text{g/mL}$ D-serine and incubated at 30 °C for 2 days. **D** Agarose gel confirmation of the presence of *dsdA*. After the transformation of the *dsdA* gene, genomic DNA was isolated, and the *dsdA* gene was amplified by PCR



could not grow in the YNB medium (without $(\text{NH}_4)_2\text{SO}_4$) at different D-serine concentrations. Then, we transformed the centromere (CEN)/autonomously replicating sequence (ARS) plasmid (YLEP-ET2-*dsdA*) expressing *dsdA* as a selectable marker (under the control of the *EXP1* promoter and the *ICL1* terminator) into *Y. lipolytica*. As shown in

Fig. 1B, when the concentration of D-serine was lower than 250 $\mu\text{g/mL}$, the transformants showed poor growth. However, transformants could grow normally when the D-serine concentration exceeded 250 $\mu\text{g/mL}$. The linear DNA fragment containing the *dsdA* expression cassette was then transformed into *Y. lipolytica*. The transformants with an

integrated *dsdA* expression cassette in the genome could grow at 500 $\mu\text{g}/\text{mL}$ D-serine in plate media (Fig. 1C). To confirm the *dsdA* expression in PO1f, 5 transformants were randomly selected from plasmid containing transformants or genomically integrated transformants. Genomic DNA was then isolated. The presence of the *dsdA* gene in *Y. lipolytica* was verified by PCR (Fig. 1D). Furthermore, we also extracted the RNA and analyzed the transcription of *dsdA* (Supplemental Table S2). The results confirmed that *dsdA* was expressed in the transformants. We also tested if *dsdA* can be used for other strains. As shown in Supplemental Fig. S3, when *dsdA* containing plasmid was transformed to Y1444 and Y31248 (the strain from the CICC), they can also grow in the minimal medium containing 500 $\mu\text{g}/\text{mL}$ D-serine (Supplemental Fig. S3). Our results showed that *dsdA* was effectively expressed and could be used as a genetic marker in *Y. lipolytica*.

Determination of the selection condition of G418, zeocin, and mycophenolic acid

In addition to *dsdA*, we also tested the applicability of several other resistance markers, which have been used in different yeast species. G418 is widely used for genetic

manipulation in *Saccharomyces cerevisiae* (Webster and Dickson 1983; Wang et al. 1996). It is reported that many prokaryotes and eukaryotes are sensitive to G418 (Colbère-Garapin et al. 1981). In *S. cerevisiae*, the *kanMX* gene coding for aminoglycoside 3-phospho-transferase (APH) confers resistance to G418 (Jimenez and Davies 1980). Here, we tested whether the drug can be used as a selectable marker in *Y. lipolytica*. The growth of PO1f was completely inhibited at a concentration of 7.2 mg/mL, which was much higher than the effective concentration in *S. cerevisiae* (Qiu et al. 2022) (Fig. 2A). We then evaluated the resistance of *kanMX* as a selectable marker by transforming the plasmids (YLEP-ET2-*kanMX*) that contain *kanMX*-transformed into PO1f. The *kanMX*-transformed cells gave rise to well-defined colonies on G418 plates. At the same time, control transformations (PO1f without *kanMX* gene) failed to form colonies (Fig. 2B). Both PCR and RT-qPCR results confirmed that the *kanMX* gene was expressed in PO1f (Fig. 2C, Supplemental Table S2, and Supplemental Fig. S2). Moreover, we demonstrated that the 7.2 mg/mL concentration of G418 also applies to screen W29 (Supplemental Fig. S4). However, the required concentration is much lower for Y1444 and Y31248 (Supplemental Fig. S5 and Supplemental Fig. S6).

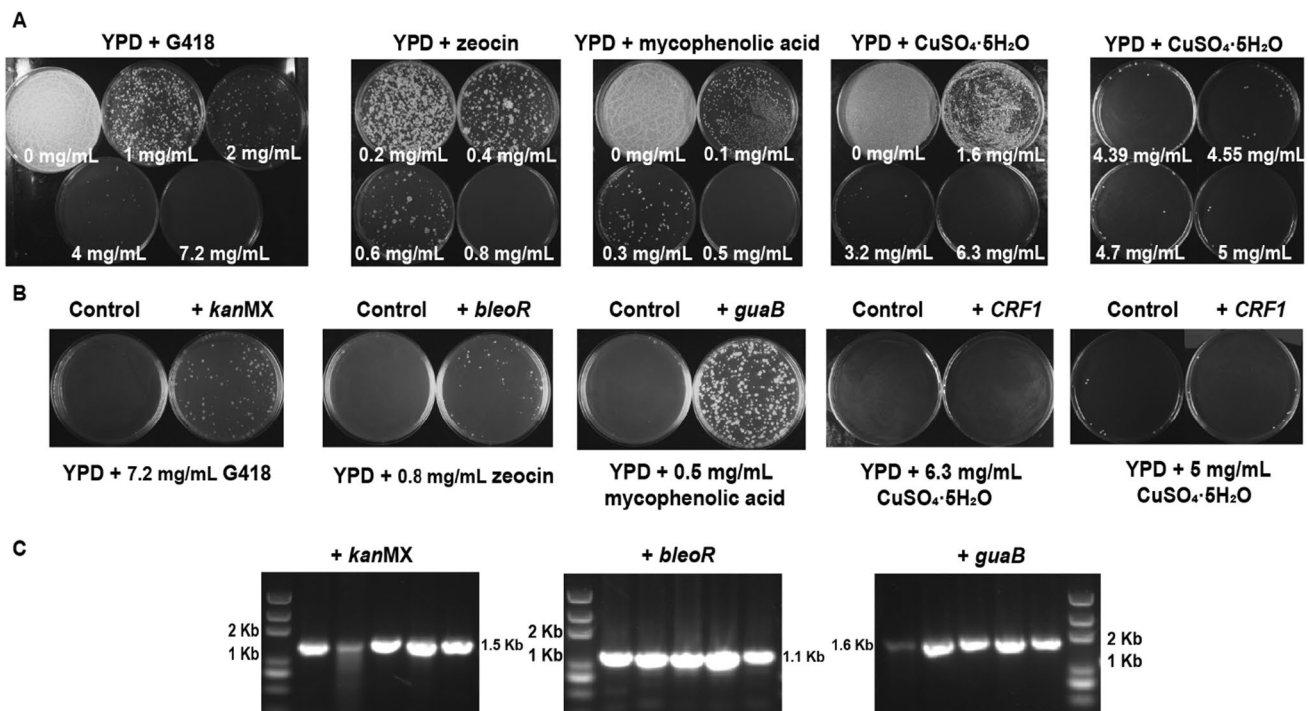


Fig. 2 Determination of the selection conditions for the dominant markers in PO1f. **A** Exploration of the drug concentration that inhibits the growth of *Y. lipolytica* strain PO1f. **B** The growth of the strain after transforming plasmids with selection-marker genes into PO1f. The transformants were plated on YPD plates (adding 7.2 mg/mL G418, 0.8 mg/mL zeocin, 0.5 mg/mL mycophenolic acid, and

6.3 mg/mL or 5 mg/mL CuSO₄·5H₂O, respectively) and incubated at 30 °C for 3 days. **C** Agarose gel confirmation of the presence of selection marker genes. After the transformation of a marker gene, genomic DNA was isolated, and the marker genes were amplified by PCR

Zeocin is a copper-chelated glycopeptide antibiotic that causes cell death by intercalating into DNA and cleaving it (Utashima et al. 2017). The *bleoR* gene from *S. hindustanus* encodes a small acidic protein (MW 13665) that binds to zeocin at a specific ratio, making it unable to bind DNA and inhibiting its DNA cleavage activity (Drocourt et al. 1990; Leiting and Noegel 1991). The resistance gene against zeocin is an excellent marker for transforming eukaryotes and prokaryotes. Tsakraklides et al. (2018) used 1 mg/mL zeocin to select transformants *Y. lipolytica* strain YB-392, while Wang et al. (2022) used 50 to 250 µg/mL bleomycin to inhibit or delay the growth of strain PO1f. In our case, we also showed that the *bleoR* conferred PO1f resistance to zeocin when the selection condition of zeocin was 0.8 mg/mL for PO1f (Fig. 2, Supplemental Table S2, and Supplemental Fig. S2). Differently, W29, Y1444, and Y31248 were completely inhibited at the concentrations of 1.2 mg/mL, 2 mg/mL, and 2 mg/mL, higher than those needed for PO1f (Supplemental Fig. S4, Supplemental Fig. S5, and Supplemental Fig. S6).

It is reported that the *guaB*-encoding inosine monophosphate dehydrogenase from *E. coli* confers mycophenolic acid resistance (Farazi et al. 1997). The first report of the dominant selection marker in *Y. lipolytica* was published by Wagner et al. (2018). The concentration of mycophenolic acid they used for PO1fΔ*TRP1* was 100 mg/L. In our experiment, we tested the different concentrations of mycophenolic acid in PO1f and found that 500 mg/L mycophenolic acid could achieve the selection of *guaB*-containing transformants of PO1f (Fig. 2, Supplemental Table S2, and Supplemental Fig. S2). Furthermore, 1700 mg/L, 2000 mg/L, and 2000 mg/L of mycophenolic acid were required to inhibit the growth of W29, Y1444,

and Y31248, respectively. These concentrations were higher than those necessary to inhibit PO1f (Supplemental Fig. S4, Supplemental Fig. S5, and Supplemental Fig. S6).

Copper confers toxicity in *Y. lipolytica*. It is reported that *Y. lipolytica* cannot grow when the concentration of CuSO_4 is higher than 22 mM (Wang et al. 2011). Wang et al. (2011) overexpressed the endogenous copper-resistance gene *CRF1* to resist higher copper concentrations (31 mM CuSO_4) and successfully selected seven transformants into the strain W29. Here, we added different concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ into the YPD medium and found that CuSO_4 could inhibit the growth of PO1f at 25 mM (6.3 mg/mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). However, when the *CRF1* gene-containing plasmids (YLEP-ET2-CRF1) were transformed into PO1f, no transformant was obtained at 25 mM CuSO_4 (Fig. 2A, B). Thus, we tried to reduce the concentration of CuSO_4 to determine whether the *CRF1* gene confers resistance to Cu^{2+} . We found that reactions with 5 mg/mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ did not inhibit cell growth completely, as there were very few colonies. Therefore, we used 20 mM (5 mg/mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) for the selection of transformants of the *CRF1* gene. However, no transformants were obtained (Fig. 2B).

Engineering the Cre/*lox* system for marker recycling

The Cre/*loxP* system is used for Cre-mediated site-specific fragment excision when two copies of the *loxP* sequence flank the fragment DNA in tandem. To avoid repeated recombination of *loxP* sites, the mutant *lox71* and *lox66* sites were introduced into constructs to transform *Corynebacterium glutamicum* (Suzuki et al. 2005a, 2007; Hu et al. 2013).

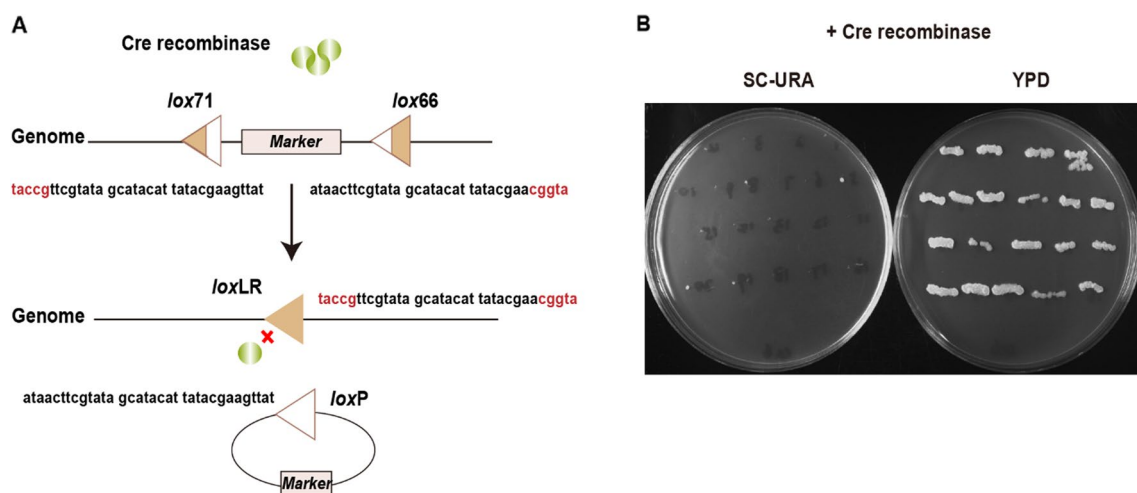


Fig. 3 The Cre/mutant *lox*-mediated marker recycling in the *Y. lipolytica* strain PO1f-URA3. **A** Recombination between a *lox66* site and a *lox71* site produces a wild-type *loxP* site within the excised DNA and a double-mutant site left in the host genome (Suzuki et al. 2005a). The double-mutant site cannot be recognized by Cre. **B** After

the expression of Cre recombinase, the *URA3* marker was efficiently removed from the genome of YLEP-Leu-Cre transformants. The left plate is a SC-URA plate while the right plate is a YPD plate. The plates were incubated at 30 °C for 3 days

The mutant *lox71* site contains mutations in the left inverted repeats of *loxP*, while *lox66* contains mutations in the right inverted repeats (Suzuki et al. 2005a). The Cre-catalyzed recombination between *lox66* and *lox71* will produce a mutant *lox* site (*loxLR*) at the place of fragment excision that is poorly recognized by Cre recombinase (Fig. 3A). In our study, in constructs for the transformation of *Y. lipolytica* (PO1f-URA3), *lox66* and *lox71* were introduced at the left and the right sides of the *URA3* marker, respectively (Supplemental Fig. S1). Cre recombinase was expressed by the transformation of the Cre-containing plasmid (YLEP-Leu-Cre). The transformants were randomly selected from PO1f-URA3 after Cre recombinase expression and streaked on YPD and SC-URA plates. As seen in Fig. 3B, no transformant could grow on a SC-URA plates. This result confirms that the Cre/mutant *lox* system can achieve marker deletion with a deletion efficiency of almost 100% (Fig. 3B).

Discussion

In this study, we developed a *dsdA* selection marker for *Y. lipolytica* and determined the selection conditions of several infrequently used markers in *Y. lipolytica*. The *dsdA* gene has previously been developed for the genetic manipulation of *S. cerevisiae* (Vorachek-Warren and McCusker 2004). When using *dsdA* as the selection marker in *S. cerevisiae*, it was reported that it allows this yeast to grow in a medium with 500 µg/mL D-serine only when expressed in a CEN/ARS plasmid. However, when *dsdA* was integrated into the genome, an additional nitrogen source (5 mg/mL L-proline and 2 mg/mL D-serine) was required. In contrast to the situation in *S. cerevisiae*, the *Y. lipolytica* strains in our study could all grow well in 500 µg/mL D-serine with either a CEN plasmid containing the *dsdA* cassette or an integrated *dsdA* cassette in the genome.

Apart from *dsdA*, we also demonstrated that the *kanMX* gene can be used as a backup selection marker in *Y. lipolytica*. However, the selection concentration of G418 was higher than in other yeasts such as *S. cerevisiae* (Qiu et al. 2022). For the antibiotic markers we tested, we found that the selection concentrations of the antibiotics for different strains (including PO1f, W29, and *Y. lipolytica* strains from CICC) were different. For example, the inhibition concentrations of zeocin and mycophenolic acid for PO1f were lower than those needed for W29 and the strains from the CICC. However, the inhibition concentrations of G418 for PO1f are higher than those required by strains from the CICC. Compared with the auxotrophic strain of PO1f, the prototrophic strains seem to have a higher tolerance to zeocin and mycophenolic acid. These findings demonstrate that the concentrations of these drugs are not the same under different conditions. Ultimately, it is necessary to explore the appropriate concentrations for each specific strain.

In contrast to the previous study, in our study, the *CRFI* gene did not confer PO1f the resistance to CuSO₄ (Wang et al. 2011). These authors used *CRFI* for gene disruption in W29, and they selected the recombinant strain in the presence of 31 mM CuSO₄. However, we did not obtain positive transformants in the presence of 20 mM CuSO₄. This may be because the expression level of the *CRFI* gene was not high enough. Wang et al. (2011) did not provide details about the promoter of the *CRFI* gene. Therefore, if the *CRFI* gene is used as the selection marker, it is necessary to test the proper expression level in the following study.

Unlike the auxotrophic markers, the dominant markers developed here do not require any modification to the host organism. Therefore, they can be easily applied to different prototrophic strains. The markers developed and optimized here expand the genetic tools available for engineering in *Y. lipolytica* and may be helpful for cell factory construction.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00253-023-12835-3>.

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Author contribution J.H. and Q.Q. conceived and designed the experiments. Y.Z. performed the experiments and the data analysis. J.L., and L.S., and M.L. contributed to data analysis. Y.Z. and J.H. wrote the manuscript. All authors read and approved the manuscript.

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Data availability This article and its supplementary information include all data generated or analyzed during this study.

Declarations

Consent for publication All authors listed in this manuscript have read and agreed to the publication of this research.

Competing interests The authors declare no competing interests.

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