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Auxotrophy-Independent Plasmid Shuttle Vectors for Applications in Diverse Yeasts

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Abstract: Plasmid shuttle vectors are a common tool used to study yeast physiology. The majority of yeast plasmids have been optimized for *Saccharomyces cerevisiae* lab strain compatibility, relying on auxotrophic complementation as their selective property. We sought to construct a series of plasmid shuttle vectors to extend functionality beyond strains with auxotrophic requirements, and test compatibility across a diverse panel of yeasts. We constructed 18 plasmids which were successfully maintained by yeasts from several genera. From a panel of 24 yeast strains, these plasmids were maintained by 18 yeasts, spanning 11 species within the genera *Lachancea*, *Metschnikowia*, *Pichia*, *Saccharomyces*, and *Torulaspora*. Additionally, an integrated gene expression reporter was assayed for functional compatibility with the 18 strains. Plasmid-derived gene expression was observed for 13 strains, spanning five species within the *Saccharomyces* genus, in addition to *Torulaspora delbrueckii*. These results indicate that this plasmid series is broadly useful for advancements and applications within academia, biotechnology, and the food and fermentation industries for research utilizing diverse *Saccharomyces* and non-*Saccharomyces* yeasts.

Keywords: yeast; plasmids; cloning; drug resistance

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1. Introduction

Saccharomyces cerevisiae is an established model organism of choice for many researchers across a broad spectrum of biological research. Characteristics of baker's yeast, such as a fully sequenced and well-characterized genome, ease of genetic manipulation, and conservation with mammalian cells, have made these unicellular fungi an ideal match for experimental eukaryotic cellular biology [1–3]. While the scientific literature on *S. cerevisiae* is plentiful, the vast majority of research has been accomplished using a subset of strains which have been genetically optimized through breeding, induced mutation, and domestication in laboratory conditions for traits to increase ease of experimental manipulation [1–3]. Despite this, rich ecological diversity exists at the strain level of *S. cerevisiae* and globally distributed *S. cerevisiae* strains have been isolated from diverse sources such as beer, wine, sake, oak, and humans [4,5]. In addition to *S. cerevisiae*, numerous non-*Saccharomyces* yeasts have been recognized for their research and biotechnological potential. While a large portion of eukaryotic microbiology research utilizes *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* has also been thoroughly studied as a model organism for molecular and cell biology [6]. Other closely related fungi, such as the heterologous protein factory *Pichia pastoris* and the riboflavin-producing *Ashbya gossypii*, have been studied and utilized for the bioprocessing of metabolites, among other topics [7,8]. To build on the academic and industrial successes of these example species, more research is needed to better understand the potential that diverse yeasts possess towards basic and applied research. One technique

with potential to accelerate our understanding of diverse yeasts and their applications is the use of plasmid shuttle vectors.

Plasmid shuttle vectors are commonly used as tools for genetic manipulation and phenotypic assessment of various microorganisms [9]. These plasmids are called shuttled vectors since they shuttle DNA between two organisms, for example, *Escherichia coli* and *S. cerevisiae*. To do so, these plasmids require origins of replications and selective markers for both host organisms [10–12]. Plasmid shuttle vectors used as expression vectors in yeast are often distinguishable based on variabilities in plasmid origins of replication, selective markers, or cloning elements. Most yeast expression vectors sets have been designed and optimized for use in lab strains of *S. cerevisiae*, limiting their applicability to more diverse yeasts [13]. The pRS series and its derivatives are popular yeast vectors which have been cited heavily in the literature across the last two decades (pRS citation count as of July 2023 = 10,161). As a brief history, Sikorski and Heiter developed the pRS series by adding selectable marker genes *HIS3*, *TRP1*, *LEU2*, and *URA3*, as well as the yeast origin of replication *CEN6/ARS4* to the pBluescript backbone [14]. Next, Christianson et al. developed a high-copy version of the low-copy pRS vectors by replacing each *CEN6/ARS4* element with the 2µ origin of replication containing the *REP3* and *FRT* sequences necessary for high-copy propagation [15]. Mumberg and Funk later introduced standardized elements to allow for expression of heterologous proteins by flanking the multiple cloning site with a variety of promoters and a 3′ untranslated region (3′UTR) containing a transcriptional terminator from *CYC1* [16]. Most recently, the pRSII series included updates from the pRS series with new biosynthetic marker genes, as well as updates to the restriction sites, removing sequence overlap with some biosynthetic markers in addition to publishing complete sequences for each vector [17].

One element of expression vectors which has been optimized for lab strains of *S. cerevisiae* is the selective marker. Often, this element either complements an auxotrophic mutation or confers drug resistance [18]. The low- and high-copy pRSII vectors utilize auxotrophic complementation for plasmid selection; therefore, these plasmids cannot be used for selection in prototrophic yeasts. Additionally, while easily tested, it is often unclear if novel strains carry any of these auxotrophic mutations, which further complicates the plasmid selection process. Specific auxotrophic mutations can be introduced to these yeasts through induced mutation or genetic engineering; however, this process can become cumbersome when numerous strains are involved [19]. Moreover, rescuing auxotrophic mutations by either genetic complementation or nutritional supplementation can result in a physiology distinct from an isogenic prototroph, further complicating strain phenotyping [20]. An alternative selective method, conferred drug resistance, does not require any auxotrophic mutations in recipient strains and only requires the host cell to be sensitive to an antifungal drug. Commonly used antifungal compounds in yeast research, such as G418, hygromycin B, and nourseothricin, result in broad spectrum cytotoxicity by inhibiting protein synthesis through ribosomal binding or mRNA/tRNA translocation interference [21,22]. As a result, fungicides have been utilized as a popular selective agent against diverse yeast species. The genomic or plasmid-based presence of a dominant drug resistance marker, or drug resistance cassette, such as kanMX, hphMX, and natMX, results in selectivity for a transgenic yeast cell when subjected to these antifungal compounds. Although these resistance cassettes are similarly structured with a flanking promoter and 3′ UTR from the *Ashbya gossypii* transcription elongation factor *TEF*, the cassettes differ in the expressed gene which confers resistance to each specific drug [23]. kanMX contains the gene *kan*^{r} which encodes for an aminoglycoside phosphotransferase to inactivate G418 (Geneticin) by ATP-mediated phosphorylation. hphMX contains the gene *hyg*^{r} which inactivates hygromycin B by a similar mechanism of action. Lastly, natMX contains the gene *nat1* which encodes for an N-acetyl transferase to inactivate nourseothricin (clonNAT) by acetylation of the beta-lysine residue [24–28]. Many studies throughout the published literature have included construction of yeast plasmids containing an anti-fungal drug-resistance marker, often to serve a unique purpose related to the research performed. Several plasmids

with various drug resistance markers including the kanMX4, bleMX4, hphMX4, natMX4 and patMX4 cassettes for G418, phleomycin, hygromycin B, nourseothricin and bialaphos resistance, respectively, were made available with the published pRSII plasmids [17]. However, these drug-resistance plasmids were intended to be used as PCR templates or for genomic integration as they did not contain a yeast origin of replication, which limits their use for plasmid-based gene expression studies.

Another element of expression vectors which has been optimized for lab strains of *S. cerevisiae* is the yeast origin of replication. Low-copy pRSII vectors utilize the *CEN6/ARS4* element as the yeast origin of replication. *CEN6* is a *S. cerevisiae* centromere and *ARS4* is the histone 4 associated autonomously replicating sequence, and together they allow the vector to be maintained through the chromosomal segregation mechanism and replicated during DNA synthesis, respectively [29–31]. Many *S. cerevisiae* strains, other *Saccharomyces* species such as *S. paradoxus*, *S. uvarum*, *S. mikatae*, and *S. kudriavzevii*, and a few non-*Saccharomyces* yeasts such as *P. kudriavzevii* and *T. delbrueckii* have proven to be effective at utilizing *CEN6/ARS4* to replicate plasmid DNA [32–36]. High-copy pRSII vectors utilize 2µ *ORI-STB* as the yeast origin of replication. 2µ plasmids are maintained at approximately 30–90 plasmid copies per cell due to Flp-mediated recombination and are randomly segregated into daughter cells [37–39]. 2µ plasmids have widespread applications across various strains of *S. cerevisiae*; however, it is unclear how functional the origin of replication is among other diverse yeasts.

While individual drug-selectable plasmids have been constructed for specific purposes and published in the scientific literature, here we sought to develop a standardized collection of characterized plasmids to increase the molecular genetic resources available to yeast researchers.

In this study, we looked to expand the toolbox of yeast gene expression systems by constructing and testing a series of auxotrophy-independent plasmid shuttle vectors. To accomplish this goal, we designed and constructed 18 novel plasmids and tested plasmid compatibility with a diverse yeast panel including conventional (*S. cerevisiae*) and non-conventional (other *Saccharomyces* spp. and non-*Saccharomyces*) yeasts. First, we replaced the auxotrophic markers on pRSII plasmid backbones with commonly used dominant drug resistance markers encoding G418, hygromycin B, and nourseothricin resistance. Second, we modified the first plasmid series by incorporating yeast expression elements including a constitutive *TDH3* promoter, multiple cloning site, and 3′ *CYC1* UTR. Last, we inserted the gene encoding the fluorescent protein mNeonGreen into the multiple cloning site of each plasmid to assess plasmid-derived protein production [40]. After plasmid construction, we sought to determine how functional the novel plasmid shuttle vectors were for a panel of diverse yeasts. A panel of 24 yeasts including various *S. cerevisiae* strains, other *Saccharomyces* species, and non-*Saccharomyces* yeasts were chosen and evaluated for their compatibility with this plasmid set. First, we screened the panel for their ability to uptake and maintain plasmid DNA using a common lithium acetate-based transformation method and quantified individual transformation efficiencies. Second, we confirmed that the selective properties of this plasmid set could be applied to our entire diverse yeast panel and assessed plasmid maintenance for each transformable tested strain. Last, we quantified plasmid-derived protein production via mNeonGreen fluorescence measurements. Taken together, this study yielded a panel of auxotrophy-independent low- and high-copy plasmid shuttle vectors to help promote future research involving diverse yeasts.

2. Methods

2.1. Strains, Media, and Culture Conditions

A diverse panel of 24 yeast strains were utilized during this study [Table 1]. The yeast panel included seven *S. cerevisiae* strains, five other *Saccharomyces* species, and 12 non-*Saccharomyces* species. Strains were obtained from multiple labs and stored as −80 °C freezer glycerol stocks. Non-transformed yeasts were cultured in YPD liquid media (1% *w*/*v* yeast extract, 2% *w*/*v* peptone, 2% *w*/*v* D-glucose) or SD liquid media (0.67% *w*/*v*

yeast nitrogen base w/o ammonium sulfate + 76 mM monosodium glutamate (MSG), 2% *w*/*v* D-glucose), and grown on YPD plates (1% *w*/*v* yeast extract, 2% *w*/*v* peptone, 2% *w*/*v* D-glucose, 2% *w*/*v* bacteriological agar). Transformant yeasts were cultured in YPD or SD liquid media supplemented with hygromycin B (333 µg/mL), G418 (300 µg/mL), or nourseothricin (100 µg/mL), unless otherwise noted, and selected for on YPD plates supplemented with identical concentrations of hygromycin B, G418, or nourseothricin. *E. coli* plasmid host cultures were cultured in LBA liquid media (2.5% *w*/*v* VWR Luria-Bertani broth powder, 100 µg/mL ampicillin) and selected for on LBA plates (2.5% *w*/*v* VWR Luria-Bertani broth powder, 100 µg/mL ampicillin, 2% *w*/*v* bacteriological agar). All liquid yeast and *E. coli* cultures were grown in 15 mL culture tubes with a 5 mL culture volume at 30 °C on a roller wheel set at 70 RPM. Plated yeast cultures were grown at 30 °C and plated *E. coli* cultures were grown at 37 °C.

Table 1. Yeast isolates used in this study.

Strain Code	Aliases	Species	Description	Source
DBY12000	FY2648	*Saccharomyces cerevisiae*	Lab strain; prototrophic derivative of S288C, MATa, HAP1+	[41]
DBY17018		*Saccharomyces uvarum*		[42]
DBY17019		*Saccharomyces mikatae*		[42]
DBY17020		*Saccharomyces kudriavzevii*		[42]
DBY17021		*Naumovozyma castellii*		[42]
DBY17022		*Lachancea kluyveri*		[42]
DBY18207	DBVPG6304	*Saccharomyces paradoxus*	American subpopulation; from California	Leonid Kruglyak lab
DBY18222	N-44	*Saccharomyces paradoxus*	Far-Eastern subpopulation; from Russia	Leonid Kruglyak lab
OYL005		*Saccharomyces cerevisiae*	Commercial beer strain; Irish Ale	Omega Yeast Labs
OYL100		*Saccharomyces pastorianus*	Commercial beer strain; Lager I	Omega Yeast Labs
PGY12	BJ20, YJS4581, OS_552	*Saccharomyces cerevisiae*	Oak isolate; from Dongling Mountain, Beijing, China	Joseph Schacherer lab
PGY34	YPS1000, YJS168	*Saccharomyces cerevisiae*	Oak isolate; from New Jersey, United States	Joseph Schacherer lab
PGY68	Y819	*Saccharomyces cerevisiae*	Commercial wine strain; Uvaferm 43	E&J Gallo Winery
PGY83	Y834	*Saccharomyces cerevisiae*	Commercial wine strain; Lalvin EC-1118	E&J Gallo Winery
PGY320	UCD7	*Pichia membranafaciens*		UC-Davis V&E Department
PGY321	UCD227	*Schizosaccharomyces pombe*	Strain FST 40-277; ATCC 2476, NRRL Y-164	UC-Davis V&E Department
PGY323	UCD601	*Lachancea thermotolerans*	Wine isolate; strain Radler 40	UC-Davis V&E Department
PGY325	UCD751	*Zygosaccharomyces bailii*	Strain PS p.194	UC-Davis V&E Department
PGY326	UCD848	*Metschnikowia pulcherrima*		UC-Davis V&E Department
PGY327	UCD1017	*Hanseniaspora uvarum*	Strain 1015-IFI	UC-Davis V&E Department
PGY328	UCD2116	*Pichia kudriavzevii*	Candida krusei; from Luna barrel fermentation	UC-Davis V&E Department

Table 1. *Cont.*

Strain Code	Aliases	Species	Description	Source
PGY329	UCD2221	*Torulaspora delbrueckii*	Wine isolate	UC-Davis V&E Department
PGY330	UCD2510	*Saccharomycodes ludwigii*	Wine isolate	UC-Davis V&E Department
PGY332	UCD2077	*Brettanomyces bruxellensis*	AWRI 1499-like strain	UC-Davis V&E Department

2.2. Optical Density to Cell Count Conversion

Liquid cultures of each transformable yeast were grown overnight in biological triplicate and subjected to various cell-counting techniques. Optical densities (OD) at λ = 600 nm were measured for each culture using a Thermo Scientific Genesys 6 spectrophotometer. Colony forming units were measured for each culture by plating serial dilutions. Cell counts were measured for each culture by counting at least 300 individual cells on a hemocytometer. Particle counts and mean particle sizes were measured for each culture using a Beckman Coulter Counter Z2. A conversion factor was generated for each yeast species/strain to easily convert measured OD_{600} values to estimated cell concentrations. Using these conversion factors, the OD values for subsequent transformation efficiency and fluorescence measurements are reported as to "per 10^6 cells".

2.3. Plasmid Construction

Plasmids used in this study can be found in Table 2. Plasmids were extracted from *E. coli* hosts with the Omega E.Z.N.A. Plasmid DNA Mini Kit I. Primers used in this study can be found in Supplemental Table S1. Primers for PCR amplicon inserts were designed with 40 base pair overhangs identical to the 5′ and 3′ ends of the linearized vector backbones. Intermediate plasmid components were purified with a GeneJET Gel Extraction Kit and confirmed by a combination of PCR, restriction enzyme digestion, and gel electrophoresis. Linearized vectors and amplicon inserts were combined using Gibson Assembly and TOP10 *E. coli* transformants were selected using LBA plates [43]. Plasmid construction was broken into three phases. (1) pRSII416 and pRSII426 vector backbones were linearized to exclude *URA3* via PCR amplification. Drug cassette inserts kanMX, hphMX, and natAC were PCR amplified from pRS416-KanMX-$TDH3_{pr}$, pRS416-HphMX-$TDH3_{pr}$, and pRS416-NatAC-$TDH3_{pr}$, respectively. Linearized pRSII416 and pRSII426 backbones were individually combined with the three drug cassette amplicons resulting in six pRSII4XD plasmids (X = origin of replication, D = drug resistance marker). (2) pRSII4XD plasmids were linearized by restriction enzyme digestion using KpnI-HF and SacI-HF (New England Biolabs). A $TDH3_{pr}$-MCS-$CYC1_{3'UTR}$ insert was PCR amplified from p416GPD; the sequence of this region can be found in Supplemental File S1, including all restriction enzyme sites for future plasmid design using these vector backbones. The six linearized pRSII4XD backbones were individually recombined with the $TDH3_{pr}$-MCS-$CYC1_{3'UTR}$ amplicon resulting in six pRSII4XD-$TDH3_{pr}$ plasmids. (3) pRSII4XD-$TDH3_{pr}$ plasmids were linearized by restriction enzyme digestion using SpeI-HF and XhoI (New England Biolabs). The gene encoding fluorescent protein mNeonGreen was PCR amplified from pK127-mNeonGreen. The six linearized pRSII4XD-$TDH3_{pr}$ backbones were individually combined with the mNeonGreen amplicon resulting in six pRSII4XD-$TDH3_{pr}$-mNeon plasmids. The resultant 18 plasmids were sequenced by Plasmidsaurus and plasmid sequences were deposited along with plasmids at Addgene (Table 2).

Table 2. Plasmids used in this study.

Name	Other Name(s)	Host	Maintenance Elements	Selective Elements	Expression Elements	Source	Addgene ID
pRSII416	RB3534	XL1-B E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Uracil auxotrophic complementation (URA3)	MCS (pBluescript II SK+)	[17]	35456
pRSII426	RB3535	XL1-B E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Uracil auxotrophic complementation (URA3)	MCS (pBluescript II SK+)	[17]	35470
pRS416-KanMX-TDH3pr	RB3398	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Uracil auxotrophic complementation (URA3)	TDH3pr-MCS-CYC13′UTR	This Study	
pRS416-HphMX-TDH3pr	RB3399	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Uracil auxotrophic complementation (URA3)	TDH3pr-MCS-CYC13′UTR	This Study	
pRS416-NatAC-TDH3pr	RB3400	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Uracil auxotrophic complementation (URA3)	TDH3pr-MCS-CYC13′UTR	This Study	
p416GPD	p416GPD	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Uracil auxotrophic complementation (URA3)	TDH3pr-MCS-CYC13′UTR	[16]	
pKT127-mNeonGreen		DH10B E.coli	*E. coli* origin (pBR322 ori)	Ampicillin-resistant (bla), G418-resistant (kanMX)	SP6pr-mNeon-ADH13′UTR	[40]	
pRSII41K	PGB74	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), G418-resistant (kanMX)	MCS	This Study	194522
pRSII42K	PGB75	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), G418-resistant (kanMX)	MCS	This Study	194523
pRSII41N	PGB80	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Nourseothricin-resistant (natAC)	MCS	This Study	194524
pRSII42N	PGB81	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Nourseothricin-resistant (natAC)	MCS	This Study	194525
pRSII41H	PGB78	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Hygromycin B-resistant (hphMX)	MCS	This Study	194526
pRSII42H	PGB79	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Hygromycin B-resistant (hphMX)	MCS	This Study	194527
pRSII41K-TDH3pr	PGB94	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), G418-resistant (kanMX)	TDH3pr-MCS-CYC13′UTR	This Study	194528
pRSII42K-TDH3pr	PGB95	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), G418-resistant (kanMX)	TDH3pr-MCS-CYC13′UTR	This Study	194529
pRSII41N-TDH3pr	PGB96	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Nourseothricin-resistant (natAC)	TDH3pr-MCS-CYC13′UTR	This Study	194530
pRSII42N-TDH3pr	PGB97	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Nourseothricin-resistant (natAC)	TDH3pr-MCS-CYC13′UTR	This Study	194531
pRSII41H-TDH3pr	PGB99	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Hygromycin B-resistant (hphMX)	TDH3pr-MCS-CYC13′UTR	This Study	194532
pRSII42H-TDH3pr	PGB98	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Hygromycin B-resistant (hphMX)	TDH3pr-MCS-CYC13′UTR	This Study	194533

Table 2. *Cont.*

Name	Other Name(s)	Host	Maintenance Elements	Selective Elements	Expression Elements	Source	Addgene ID
pRSII41K-TDH3pr-mNeon	PGB100	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), G418-resistant (kanMX)	TDH3pr-mNeon-CYC13′UTR	This Study	194534
pRSII42K-TDH3pr-mNeon	PGB101	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), G418-resistant (kanMX)	TDH3pr-mNeon-CYC13′UTR	This Study	194535
pRSII41N-TDH3pr-mNeon	PGB102	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Nourseothricin-resistant (natAC)	TDH3pr-mNeon-CYC13′UTR	This Study	194536
pRSII42N-TDH3pr-mNeon	PGB103	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Nourseothricin-resistant (natAC)	TDH3pr-mNeon-CYC13′UTR	This Study	194537
pRSII41H-TDH3pr-mNeon	PGB105	TOP10 E.coli	*E. coli* origin (pBR322 ori), Low-copy (CEN6/ARS4)	Ampicillin-resistant (bla), Hygromycin B-resistant (hphMX)	TDH3pr-mNeon-CYC13′UTR	This Study	194538
pRSII42H-TDH3pr-mNeon	PGB104	TOP10 E.coli	*E. coli* origin (pBR322 ori), High-copy (2μ ORI-STB)	Ampicillin-resistant (bla), Hygromycin B-resistant (hphMX)	TDH3pr-mNeon-CYC13′UTR	This Study	194539

2.4. Evaluation of Transformation Efficiency

All yeast transformations were performed using a standard lithium acetate method [44]. Briefly, yeast cultures were grown overnight to saturation, culture optical densities were measured, and cells were subcultured to a starting OD_{600} of 0.05. After approximately 6 h of growth on a roller wheel at 30 °C, 4 mL of cells were collected and washed with sterile water then TE/LiAc solution (10 mM Tris-HCl, 1 mM EDTA, 0.5 M LiAc). Washed cells were resuspended in 70 μL of TE/LiAc solution, then 10 μL of boiled salmon sperm DNA, 60 ng of plasmid DNA, and 720 μL of PEG/TE/LiAc solution (10 mM Tris-HCl, 1 mM EDTA, 0.5 M, 40% PEG_{3350}) were added to the cell suspension. Next, cells were incubated at 30 °C for 30 min, then 73 μL of DMSO was added and cells were heat shocked at 37 °C for 15 min. Cells were pelleted, resuspended in 250 μL of sterile water, serially diluted, and plated onto YPD. After 24 h of recovery growth, transformants were replica plated onto selective media. Using a single plasmid preparation for all transformations, transformations were performed in biological triplicate, and transformation efficiencies were calculated for all 24 yeast strains. Yeasts which did not yield transformants were excluded from future transformation-based experiments. The six pRSII4XD-$TDH3_{pr}$-mNeon plasmids were individually transformed into all transformable yeast strains. Transformation efficiencies were calculated for each strain-plasmid combination with one biological replicate. Strain-plasmid combinations which did not yield transformants on the first attempt were transformed at least three times to distinguish between low transformation efficiency and strain-plasmid incompatibility.

2.5. Evaluation of Plasmid Maintenance in Selective and Non-Selective Media

Low- and high-copy transformants were passaged by sequentially re-streaking isolated colonies onto selective media. Viable re-streaks were determined by the number of times a transformant could be re-streaked onto selective media before the drug resistance phenotype was lost. Transformants were re-streaked a maximum of five times. Low- and high-copy transformants of *S. cerevisiae* (lab) were grown overnight in non-selective media, plated onto YPD, and after 24 h of recovery growth, transformants were replica plated onto selective media. Colony counts were compared between selective and non-selective media to calculate % drug-resistant colonies.

2.6. mNeonGreen Fluorescence Measurements

Non-transformed strains, low-copy transformants, and high-copy transformants were grown in liquid media until cultures reached the stationary growth phase. Culture OD_{600} was measured, 1 mL of cells were washed once in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and resuspended in 1 mL of PBS. Washed cells were loaded into a Corning Costar 96-Well Black Polystyrene Plate and three tenfold serial dilutions were made in PBS, leaving a final volume of 90 µL in each well. End point fluorescence was measured at excitation and emission wavelengths of 492 nm and 527 nm, respectively, using the Gen5 software on a BioTek Synergy H1 Hybrid Reader. Background fluorescence was measured for non-transformed overnight cultures and used as background for each transformant fluorescence value. Biological triplicates were used to calculate average fluorescence per 10^6 cells.

3. Results & Discussion

3.1. Design and Construction of 18 Drug-Selectable Yeast Plasmid Vectors

Plasmids pRSII416 and pRSII426 were used as vector backbones for the plasmids constructed here and are identical apart from their origin of replication. pRSII416 is a low-copy vector with *CEN6/ARS4* maintaining plasmid at chromosomal levels, whereas pRSII426 is a high-copy vector with the 2µ element maintaining plasmid at increased levels (30–90 copies per cell). The increase in plasmid copy number generally results in an increase in expression levels for the genes encoded on these plasmids. The original design of these plasmids relies on uracil auxotrophy complementation as the selective pressure for plasmid maintenance. While auxotrophy-dependent selective pressure is common for plasmid shuttle vectors, this plasmid maintenance approach requires the host yeast strain to carry specific auxotrophic mutations, often in nucleotide or amino acid biosynthetic genes, which restricts the list of readily compatible yeast host strains. By constructing plasmids with an alternative selection method, drug resistance, the spectrum of compatible yeast species and strains can be expanded. A total of 18 novel drug-selectable yeast expression vectors were successfully constructed during this study [Figure 1]. The first round of plasmid construction involved replacing the auxotrophic marker *URA3* on a pRSII4X6 vector backbone with one of three drug-resistance-encoding cassettes: kanMX, hphMX, or natAC. For each drug marker, a low- and high copy version was constructed, resulting in six pRSII4XD (X = origin of replication, D = drug resistance marker) plasmids. Plasmid sequence analysis confirmed replacement of *URA3* with selected drug markers.

Plasmid shuttle vectors are often used to study DNA inserts in a highly controlled setting. To successfully express genes, plasmid shuttle vectors typically contain a region which includes a promoter, MCS, and a 3′ UTR. To ensure gene expression is constant, a constitutive promoter can be utilized. The next round of plasmid construction involved cloning a strong, constitutive promoter from the *TDH3* gene, an MCS, and a 3′ UTR from the *CYC1* gene into the first six plasmids from this study. A PCR amplicon of $TDH3_{pr}$-MCS-$CYC1_{3'UTR}$ was cloned into the original MCS of the first six plasmids, yielding six pRSII4XD-$TDH3_{pr}$ plasmids. DNA sequence analysis confirmed successful integrations of this amplicon into each plasmid. To confirm the functionality of the integrated $TDH3_{pr}$-MCS-$CYC1_{3'UTR}$ element, a gene sequence encoding a monomeric fluorescent protein mNeonGreen was cloned into the MCS of the pRSII4XD-$TDH3_{pr}$ plasmids, yielding six pRSII4XD-$TDH3_{pr}$-mNeon plasmids designed to constitutively express mNeonGreen. DNA sequence analysis confirmed successful integrations of this amplicon into each plasmid. Each of the newly constructed plasmids have been deposited in the Addgene plasmid repository and have been assigned unique Addgene accession numbers [Table 2]. The GenBank sequence file and corresponding plasmid maps for the novel plasmids can also be found on Addgene in addition to Supplemental Figure S1.

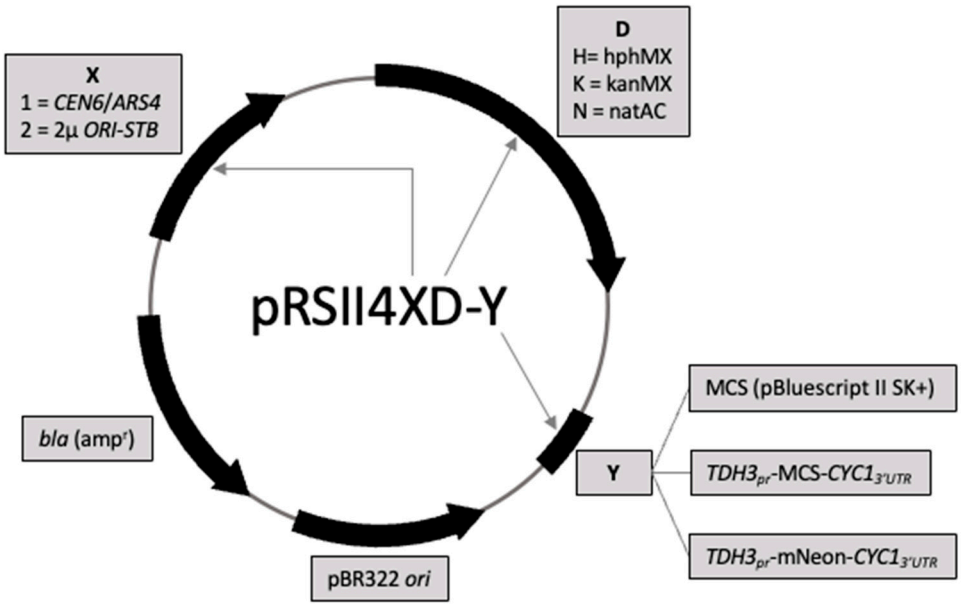

Figure 1. Features of the 18 yeast plasmids constructed in this study. Plasmids constructed as low-copy or high-copy versions, distinguishable by the yeast origin of replication present in region "X". Three different versions of low-copy and high-copy plasmids contain different dominant drug resistance markers present in region "D". Plasmids are also distinguishable by the MCS region "Y". All 18 plasmids contain *bla* and *ori* for bacterial selection and maintenance, respectively.

3.2. Transformation Efficiencies Are Highly Variable across the Diverse Yeast Panel

The 18 plasmids constructed in this study rely on conferred drug resistance as the selective pressure for plasmid maintenance and as a result, the four requirements for a yeast strain to maintain these plasmids are native antifungal drug sensitivity, transformability, recognition and usage of the plasmid origin of replication, and ability to express a functional drug resistance protein using the *A. gossypii TEF* gene promoter. Sensitivity to the three antifungal drugs was confirmed for the 24 yeast strains by pronging serial dilutions of each strain onto YPD plates supplemented with hygromycin B, G418, or nourseothricin. Growth on drug-supplemented plates was not observed. The selective pressures to maintain the novel plasmids were confirmed for the 24 strains. However, it was unclear if each strain could uptake and maintain foreign DNA via transformation. There are various approaches to transforming yeast strains with foreign DNA including the spheroplast method, lithium acetate method, electroporation, biolistic method, and glass bead method [45]. Due to the simplicity and cost effectiveness of lithium acetate chemical transformations, we utilized a version of this method across the panel of 24 strains. Transformation efficiencies for the 24 drug-sensitive yeast strains were determined using a single low-copy vector, pRSII41H-*TDH3*$_{pr}$-mNeon. The transformation efficiency results for each strain were categorized into five transformation efficiency score groups: none, low, medium, high, and very high [Table 3]. Six of the 24 strains did not yield transformants, and notably, all six strains were from non-*Saccharomyces* genera. Physiological variations between microorganisms which reduce the success of the lithium acetate method may have prevented transformant colonies from growing. Additionally, the *Ashbya gossypii TEF* promoter, which controls the expression for all of the drug resistance cassettes, may not have been functional for all the strains tested. *Metschnikowia pulcherrima* was classified as low efficiency. However, only one of three biological replicates yielded transformant colonies. *Saccharomyces pastorianus* (beer, Lager I), *Saccharomyces cerevisiae* (beer, Irish Ale), *Pichia kudriavzevii*, and *Pichia membranifaciens* also yielded low transformation efficiencies. *Saccharomyces cerevisiae* (wine, Uvaferm 43), *Saccharomyces paradoxus* (Far Eastern subpopulation), *Torulaspora delbrueckii*, *Saccharomyces uvarum*, and *Saccharomyces paradoxus* (American subpopulation) yielded medium transformation efficiencies. *Saccharomyces mikatae*, *Lachancea thermotolerans*, *Saccharomyces cerevisiae* (wine,

EC1118), *Saccharomyces cerevisiae* (oak, China), *Lachancea kluyveri*, and *Saccharomyces cerevisiae* (oak, NJ) yielded high transformation efficiencies. Lastly, *Saccharomyces cerevisiae* (lab) and *Saccharomyces kudriavzevii* yielded very high transformation efficiencies [Table 3]. As the lithium acetate transformation protocol was developed and optimized for transforming strains of *S. cerevisiae*, it is unsurprising that *S. cerevisiae* and other *Saccharomyces* spp. strains transformed successfully with medium to very high efficiencies while non-*Saccharomyces* yeasts had lower efficiencies. Overall, the list of drug sensitive yeast strains capable of transforming foreign DNA via the lithium acetate method reduced the yeast panel from 24 to 18 strains. It should be noted that other transformation methods may have yielded different transformation efficiencies than the lithium acetate method. Various *S. cerevisiae* strains, *S. paradoxus*, *M. pulcherrima*, and *P. kudriavzevii* have previously been transformed via the lithium acetate transformation method and *S. uvarum*, *L. kluyveri*, and *T. delbrueckii* have previously been successfully transformed via electroporation [46–48].

Table 3. Transformation efficiency of 24 diverse yeast isolates.

Yeast	Transformation Efficiency † ± (SD) [CFU/10^6 cells/µg DNA]	Efficiency Score ‡
S. cerevisiae (lab)	3984.09 (±497.32)	VERY HIGH
S. kudriavzevii	1094.97 (±426.13)	VERY HIGH
S. mikatae	535.73 (±356.49)	HIGH
L. thermotolerans	434.72 (±302.61)	HIGH
S. cerevisiae (wine, EC1118)	257.85 (±180.42)	HIGH
S. cerevisiae (oak, China)	202.11 (±197.35)	HIGH
L. kluyveri	126.56 (±110.13)	HIGH
S. cerevisiae (oak, NJ)	109.23 (±94.85)	HIGH
S. cerevisiae (wine, Uvaferm 43)	99.01 (±75.99)	MEDIUM
S. paradoxus (Far-Eastern subpopulation)	61.91 (±2.76)	MEDIUM
T. delbrueckii	57.96 (±36.28)	MEDIUM
S. uvarum	53.10 (±17.25)	MEDIUM
S. paradoxus (American subpopulation)	36.13 (±36.57)	MEDIUM
S. pastorianus (beer, Lager I)	6.47 (±8.15)	LOW
S. cerevisiae (beer, Irish Ale)	3.36 (±3.41)	LOW
P. kudriavzevii	0.39 (±0.34)	LOW
P. membranafaciens	0.35 (±0.60)	LOW
M. pulcherrima	0.10 (±0.17)	LOW
N. castelli	0	NONE
H. uvarum	0	NONE
Z. bailii	0	NONE
B. bruxellensis	0	NONE
S. pombe	0	NONE
S. ludwigii	0	NONE

† Transformation efficiency (TE) calculated by the following equation: TE = (CFU)/(10^6 cells)/(µg plasmid DNA). ‡ Efficiency score categories: very high (TE > 1000), high (TE > 100), medium (TE > 10), low (TE < 10), and none. Note: Transformations were performed using pRSII41H-*TDH3*pr-mNeon.

3.3. Yeast Origin of Replication Determines Yeast-Plasmid Compatibility

Once drug sensitivity and transformability were confirmed for these 18 strains, we sought to determine their compatibility with both low- and high-copy number yeast origins of replication. Transformations of pRSII41H-*TDH3*$_{pr}$-mNeon, pRSII41K-*TDH3*$_{pr}$-mNeon, and pRSII41N-*TDH3*$_{pr}$-mNeon yielded transformants for the entire yeast panel [Supplemental Table S3]. These results were expected as the 18 strains passed the initial transformation screening using a low-copy vector as the transforming DNA. Transformations of pRSII42H-*TDH3*$_{pr}$-mNeon, pRSII42K-*TDH3*$_{pr}$-mNeon, and pRSII42N-*TDH3*$_{pr}$-mNeon yielded transformants for 16 of the 18 strains [Supplemental Table S3]. Triplicate attempts to obtain high copy transformants for *L. kluyveri* and *M. pulcherrima* were unsuccessful, suggesting incompatibility between these species and the *S. cerevisiae* 2μ origin of replication present on the high-copy vectors. Possible explanations for the observed incompatibility include a lack of recognition or usage of the 2μ element to replicate DNA, or alternatively, cytotoxicity from the increased levels of plasmids or gene products encoded on the plasmids. In addition to comparing the two types of origin of replication with the strain panel, these results also highlighted that the choice of drug resistance marker on low- and high-copy plasmids did not heavily influence transformation efficiencies [Supplemental Table S3]. While the growth of colonies on selective media after transformation is a good indicator of plasmid compatibility, we sought to further elucidate the strain-plasmid relationship by testing plasmid maintenance after multiple generations of cell division. A culturing passage revealed that the 18 strains which successfully transformed could maintain the low-copy plasmids for at least five re-streaks on selective media, indicative of approximately 50–100 generations (assuming each colony consists or 10–20 generations per founding mother cell), whereas the high-copy plasmids were not maintained equally by all yeast species [Table 4]. High-copy transformants of *T. delbrueckii* and *P. kudriavzevii* successfully grew for one re-streak but failed to propagate on selective media after subsequent re-streaks. The inability to grow on selective media after multiple re-streaks indicates a loss of drug resistance and likely an inability to maintain the plasmid after multiple generations due to compatibility issues with the 2μ origin of replication.

Table 4. Plasmid maintenance for 18 diverse yeast isolates.

	Viable Re-Streaks (Maximum of 5)	
Yeast	**Low Copy**	**High Copy**
S. cerevisiae (lab)	5	5
L. thermotolerans	5	5
S. mikatae	5	5
S. cerevisiae (wine, EC1118)	5	5
S. kudriavzevii	5	5
S. cerevisiae (oak, China)	5	5
S. cerevisiae (oak, NJ)	5	5
S. cerevisiae (wine, Uvaferm 43)	5	5
S. paradoxus (Far-Eastern)	5	5
S. uvarum	5	5
S. paradoxus (American)	5	5
S. pastorianus (beer, Lager I)	5	5
S. cerevisiae (beer, Irish Ale)	5	5
P. membranafaciens	5	5

Table 4. *Cont.*

	Viable Re-Streaks (Maximum of 5)	
Yeast	**Low Copy**	**High Copy**
T. delbrueckii	5	1
P. kudriavzevii	5	1
L. kluyveri	5	not tested
M. pulcherrima	5	not tested

To evaluate plasmid maintenance after growth in various non-selective media, overnight propagation of low-copy transformants of *S. cerevisiae* (lab), a strain which has been domesticated to thrive in laboratory conditions, reduced the percent G418-resistant cells in the population to 54.70% and 54.10% for minimal and rich media, respectively [Supplemental Table S4]. Comparatively, non-selective liquid overnight propagation of high-copy transformants of *S. cerevisiae* (lab) reduced the percent G418-resistant cells in the population to 9.66% and 18.83% for minimal and rich media, respectively. The percent G418-resistant cells after propagation in non-selective media roughly translates to the proportion of plasmid-retaining cells remaining in the population. As approximately 100% of viable cells growing in selective media are expected to contain plasmids, any reduction in the percent G418-resistant cells after a lack of selective pressure indicates plasmid loss. While there was evident plasmid loss for low-copy transformants, the rate of plasmid loss for high-copy transformants was much higher, highlighting the importance of maintaining plasmid selection, especially for high-copy plasmids.

3.4. Plasmid-Derived Gene Expression Levels Vary across the Diverse Yeast Panel

After evaluating each strain's ability to uptake and maintain the plasmids, we sought to determine if the gene expression system integrated in the pRSII4XD-*TDH3*$_{pr}$ plasmids was compatible with the diverse yeast strain panel. To test this, we utilized the pRSII4XD-*TDH3*$_{pr}$-mNeon plasmids designed to constitutively express the monomeric fluorescent protein mNeonGreen. The pRSII4XD-*TDH3*$_{pr}$-mNeon plasmids allowed us to compare mNeonGreen fluorescence as a proxy for gene expression under the control of the *TDH3* promoter and relative protein expression level. The peak excitation and emission wavelengths for mNeonGreen are 506 nm and 517 nm, respectively. However, 492 nm and 527 nm were the excitation and emission wavelengths which reduced excessive background fluorescence for mNeonGreen with our instrumentation [40]. Relative fluorescence was independent of the growth phase at the time of sample collection (e.g., log or stationary phase) [Supplemental Figure S2B] and was independent of the drug used as the selective pressure for plasmid maintenance [Supplemental Figure S2C]. These observations were expected as *TDH3*$_{pr}$-driven gene expression is constitutive, resulting in mNeonGreen expression which is typically insensitive to culturing conditions or growth phase. Relative to the background fluorescence measured for the 12 non-transformed *Saccharomyces* species, fluorescence levels ranged 60–250 times higher for low-copy transformants and 25–160 times higher for high-copy transformants [Figure 2A]. The general increase in measured fluorescence for low- and high-copy transformants compared to non-transformed strains indicates that *TDH3*$_{pr}$ is a functional promoter for all of the *Saccharomyces* species assessed. Thus, the current set of pRSII4XX-*TDH3*$_{pr}$ plasmids are compatible with many *Saccharomyces* species for gene expression studies. Low- and high-copy transformants of *M. pulcherrima* did not successfully grow in selective media so mNeonGreen fluorescence could not be assessed. Additionally, *P. membranifaciens* and *L. thermotolerans* were the only non-*Saccharomyces* high-copy transformants which successfully grew in liquid selective media. Relative to the background fluorescence measured for the non-transformed non-*Saccharomyces* species, fluorescence levels were only significantly increased for low-copy transformants of *T. delbrueckii*. The increase in measured fluorescence for low-copy

transformants compared to non-transformed strains indicates that $TDH3_{pr}$ is a functional promoter for *T. delbrueckii*, however only a 10× increase in mNeonGreen fluorescence was observed [Figure 2B]. An increase in mNeonGreen fluorescence was not observed for low-copy transformants of *L. kluyveri*, *P. membranifaciens*, *L. thermotolerans*, and *P. kudriavzevii*, and high-copy transformants of *P. membranifaciens* and *L. thermotolerans*, suggesting that $TDH3_{pr}$ is non-functional for each of these species regardless of plasmid origin of replication. These results indicate that the current set of pRSII4XX-$TDH3_{pr}$ plasmids may not be compatible with some non-*Saccharomyces* species for gene expression studies due to promoter incompatibility. However, the design of these plasmids allows for a simple restriction-enzyme-mediated promoter swap which streamlines future work in revealing compatible constitutive promoters with these non-*Saccharomyces* species. This study indirectly validated *A. gossypii TEF* promoter compatibility with all of the transformable strains due to the observed acquisition of drug-resistance under the expression of the *A. gossypii TEF* promoter. Therefore, future work utilizing these plasmids with *TDH3*-incompatible yeasts can replace the *TDH3* promoter with *A. gossypii TEF* promoter or other functional promoters to ensure gene expression. It was notable that high-copy plasmid transformants yielded lower average fluorescence for all but one *S. cerevisiae* strain (beer, Irish Ale). We questioned whether the presence of the antifungal drugs impacted mNeonGreen fluorescence levels in high-copy transformants. For *S. cerevisiae* (lab), a strain with significantly higher fluorescence in low-copy transformants relative to high-copy transformants, overnight growth in non-selective minimal media and non-selective rich media did not reverse this pattern, suggesting the presence of drugs were not responsible for these unexpected trends [Supplemental Figure S3]. These results prompted an investigation into the sequence of the 2μ element found on the constructed plasmids. Sequence analysis revealed a four-nucleotide insertion in the 2μ element compared to the pRSII426 sequence published on Addgene. Additional sequencing of the original pRSII426 backbone plasmid revealed the exact four-nucleotide insertion was present. It is unclear if the observed trends in mNeonGreen expression are a result of this sequence dissimilarity, though this 2μ origin is functional for plasmid replication and maintenance as evident by repeated propagation for multiple strains in selective media [Table 4]. One possible explanation for the observed reduction in mNeonGreen fluorescence for high-copy transformants is an occurrence of plasmid burden, which has been previously shown to reduce growth rate and expression of reporter genes for transformants under similar conditions [49,50]. As mNeonGreen expression is controlled by the strong constitutive *TDH3* promoter, protein/metabolic burden can occur. For future studies utilizing these high-copy expression vectors, the *TDH3* promoter can be replaced with a weaker constitutive promoter or inducible promoter to potentially increase reporter gene expression for high-copy transformants relative to their low-copy counterparts.

In total, we constructed a series of plasmid shuttle vectors which extends usage beyond strains with auxotrophic requirements and tested plasmid compatibility across a diverse panel of yeasts. We constructed 18 total plasmids which were successfully maintained by yeasts from several genera. From a panel of 24 yeast strains, these plasmids were maintained by 18 yeasts, spanning 11 species within five different genera. Additionally, the integrated gene expression system yielded plasmid-derived protein production for 13 strains, spanning five species within the *Saccharomyces* genera, in addition to one non-*Saccharomyces* yeast. These results indicate that this plasmid series has broad applicability for various diverse *Saccharomyces* and non-*Saccharomyces* yeasts.

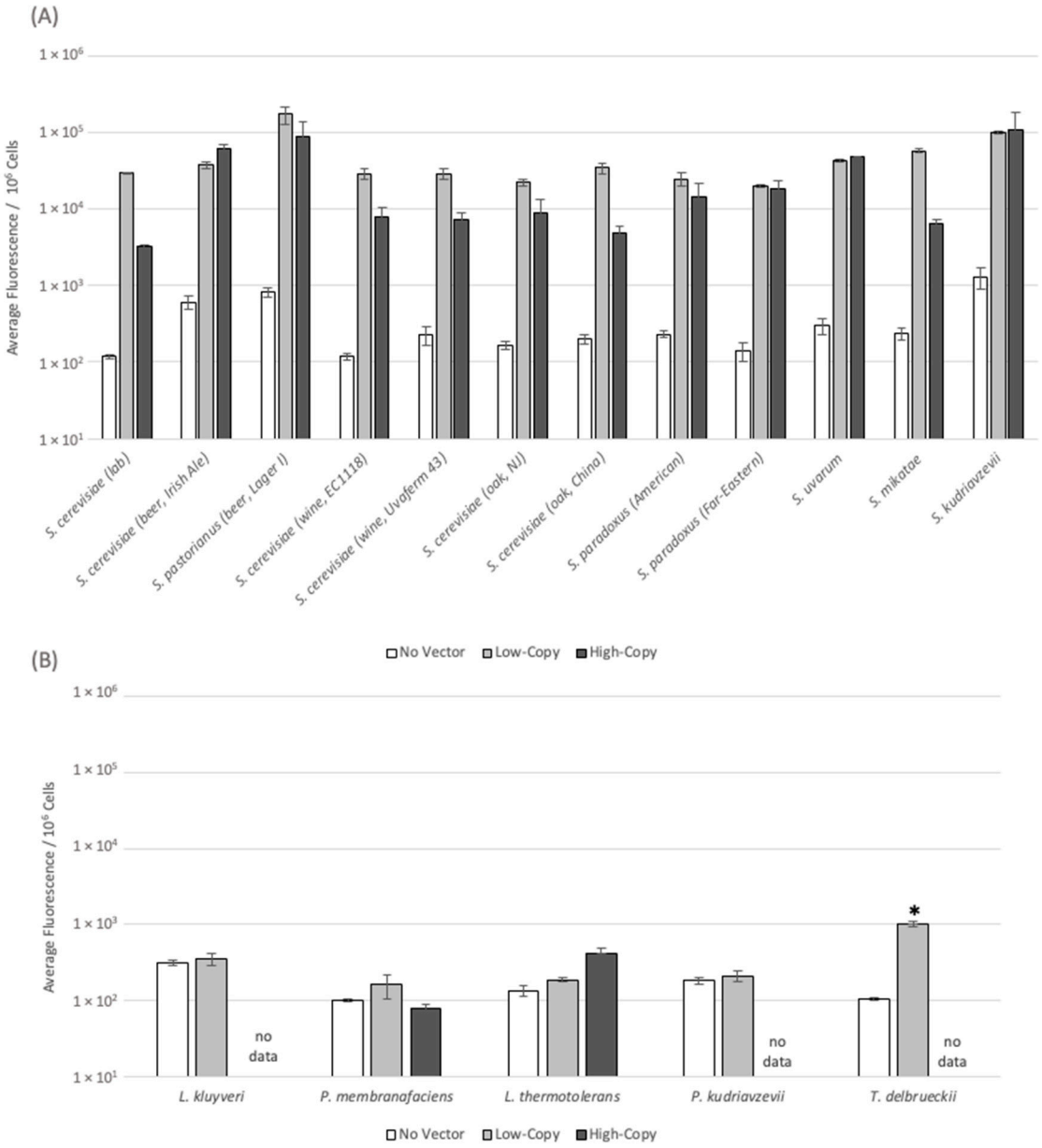

Figure 2. mNeonGreen fluorescence for Saccharomyces and non-Saccharomyces transformants. (**A**) mNeonGreen fluorescence for 12 Saccharomyces strains transformed with pRSII41K-TDH3pr-mNeon and pRSII42K-TDH3pr-mNeon. Average fluorescence/10^6 cells are shown as the average of three biological replicates with standard deviation included. (**B**) mNeonGreen fluorescence for five non-*Saccharomyces* yeast strains transformed with pRSII41K-TDH3pr-mNeon and pRSII42K-TDH3pr-mNeon. *Lachancea kluyveri* and *Pichia kudriavzevii* were unable to maintain high-copy vectors and thus, were unable to grow in liquid selective media. Asterisks represent statistical difference ($p < 0.05$) between non-Saccharomyces plasmid transformants and their non-transformed strain background fluorescence. Average fluorescence/10^6 cells is shown as the average of three biological replicates with standard deviation included.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4010031/s1, Figure S1: Plasmid maps for all 18 constructed vectors. (a) Series one (pRSII4XD) plasmids. High- and low-copy plasmids with pBluescript II SK+ MCS. (b) Series two (pRSII4XD-TDH3pr) plasmids. High- and low-copy plasmids contain the same elements as series one plasmids, with an additional *TDH3* promoter and *CYC1* 3′UTR flanking the 5′ and 3′ ends of the MCS, respectively. (c) Series three (pRSII4XD-TDH3pr-mNeon) plasmids. High- and low-copy plasmids contain the same elements as series two plasmids with mNeonGreen

cloned into the MCS. All 18 plasmids contain *bla* and *ori* for bacterial selection and maintenance, respectively. Plasmid within a series is differentiated by the yeast origin or replication (*CEN6/ARS4* or 2μ ORI-STB) and/or the drug resistance cassette (hphMX, kanMX, or natAC). All plasmid maps were designed from GenBank files using ApE. (https://jorgensen.biology.utah.edu/wayned/ape/); Figure S2: mNeonGreen fluorescence protocol optimization. (A) mNeonGreen fluorescence for *S. cerevisiae* (lab) transformed with pRSII41K-$TDH3_{pr}$-mNeon and pRSII42K-$TDH3_{pr}$-mNeon in biological triplicate. (B) mNeonGreen fluorescence during different growth phases. *S. pastorianus* (beer, Lager I) transformed with pRSII41K-$TDH3_{pr}$-mNeon and pRSII42K-$TDH3_{pr}$-mNeon, and *S. paradoxus* (American) transformed with pRSII42K-$TDH3_{pr}$-mNeon collected during exponential growth (OD_{600} < 0.500) and during stationary phase (OD_{600} > 2.00). Data shown is from a single replicate. (C) mNeonGreen fluorescence for *S. cerevisiae* (lab) transformed with low-copy plasmid variants with different drug resistance genes. Average fluorescence/10^6 cells were calculated using three biological replicates with standard deviation included; Figure S3: mNeonGreen fluorescence after overnight growth in different non-selective media. mNeonGreen fluorescence for *S. cerevisiae* (lab) transformed with pRSII41K-TDH3pr-mNeon and pRSII42K-TDH3pr-mNeon and grown in SD, YPD, SD+G418, and YPD+G418. SD contained MSG as the sole nitrogen source to accommodate for the proposed mechanism-of-action of G418 (transport through an ammonium-repressed transport protein). Average fluorescence/10^6 cells were calculated using three biological replicates with standard deviation included; Table S1: Primers used in this study; Table S2: Conversion chart for 1 OD unit to different cell counting techniques; Table S3: Transformation efficiencies for 18 diverse yeast isolates using the six mNeon plasmids; Table S4: Plasmid retention after overnight propagation in non-selective media.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Plasmids and their respective sequences can be obtained through Addgene (see Table 2 for Addgene ID numbers).

Conflicts of Interest: The authors declare no conflicts of interest.

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