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Research Article

A set of genetically diverged *Saccharomyces cerevisiae* strains with markerless deletions of multiple auxotrophic genes

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Abstract

Genome analysis of over 70 *Saccharomyces* strains revealed the existence of five groups of genetically diverged *S. cerevisiae* wild-type isolates, which feature distinct genetic backgrounds and reflect the natural diversity existing among the species. The strains originated from different geographical and ecological niches (Malaysian, West African, North American, Wine/European and Sake) and represent clean, non-mosaic lineages of *S. cerevisiae*, meaning that their genomes differ essentially by monomorphic and private SNPs. In this study, one representative strain for each of the five *S. cerevisiae* clean lineages was selected and mutated for several auxotroph genes by clean markerless deletions, so that all dominant markers remained available for further genetic manipulations. A set of 50 strains was assembled, including eight haploid and two diploid strains for each lineage. These strains carry different combinations of *leu2Δ0*, *lys2Δ0*, *met15Δ0*, *ura3Δ0* and/or *ura3Δ::KanMX-barcoded* deletions with marker configurations resembling that of the BY series, which will allow large-scale crossing with existing deletion collections. This new set of genetically tractable strains provides a powerful tool kit to explore the impact of natural variation on complex biological processes. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: **yeast; auxotrophic marker; mutant strains; genetic backgrounds**

Introduction

Yeast, such as *S. cerevisiae*, has been a powerful tool in eukaryotic genetics, from the description of the information-carrying genome to the association of gene mutation with human disorders. A huge amount of the genetic information that was gained through the study of *S. cerevisiae* has resulted, more or less directly, from the development of large-scale mutant libraries (Giaever *et al.*, 2002; Mnaimneh *et al.*, 2004; Winzeler *et al.*, 1999; Yu *et al.*, 2006), which initially relied on the design of a set of laboratory strains carrying multiple markerless deletions of auxotrophic genes (Brachmann *et al.*, 1998). These strains have allowed decisive progress to be made in our understanding of many complex biological processes, including cell metabolism, the genetics of mitochondria, meiosis, replication, DNA repair, environmental adaptation and evolution (Botstein and Fink, 2011). However, the extensively studied laboratory strains are not representative of the genetic and phenotypic diversity of the species. Remarkably, extensive cell proliferation phenotyping under many environmental conditions has indicated extreme phenotypes for the laboratory strain S288c when compared to dozens of other *S. cerevisiae* strains (Warringer *et al.*, 2011). An additional striking example of the S288c peculiarity is the extremely high rate of petite formation, which can affect multiple phenotypes (Dimitrov *et al.*, 2009).

Furthermore, recent studies have shown that the same mutation in closely related strains can lead to drastic phenotypic differences as a consequence of background-specific genetic interactions (Chin *et al*., 2012; Dowell *et al*., 2010). It is therefore essential to look at diverse backgrounds in order to pursue a comprehensive investigation of genetic polymorphism addressing, especially, how genetic combinations and contexts impact phenotypes. Furthermore, the possibility of exploiting this natural genetics variation is of extreme importance, given the emergence of *S. cerevisiae* as a model to study natural variation, ecology and population-level genetics (Cromie *et al*., in press; Hyma and Fay, 2013; Stefanini *et al*., 2012).

Genome sequencing of over 70 *Saccharomyces* strains and the study of the phylogenetic relationships within the *S. cerevisiae* population has revealed five major groups of wild-type isolates collected from diverse sources and referred to as the Malaysian, West African, North American, Wine/European and Sake groups (Liti *et al*., 2009). These groups were considered to be distinct 'clean' lineages characterized by monomorphic and private SNPs and displayed similar phylogenetic relationships across their entire genome, which contributes to forming consistent groups. The population genomics survey also revealed other strains with high sequence similarities despite distinct worldwide distribution and ecological situations (Liti *et al*., 2009). Indeed, the domestication of *S. cerevisiae* strains, e.g. selection and manipulation for human purposes, may have triggered genetic exchanges through strain crosses. Such populations with biased genomic variability, also named 'mosaic' strains, might therefore provide only a limited understanding of the relationship between the genetic background and the function, stability, dynamics and evolution of genomes. In contrast, the five groups feature distinct genetic backgrounds as a reflection of the natural diversity existing among *S. cerevisiae* species. They represent ideal tools regarding a wide variety of genetic experiments that aim to connect phenotypes to genotypes. This includes the generation of mapping populations for quantitative trait loci analysis (Cubillos *et al*., in press) and understanding trait divergence underlain by differences in expression levels (Chang *et al*., 2013; Lee *et al*., in press) and non-genetic determinants (Ziv *et al*., 2013).

Representative isolates of each of the five *S. cerevisiae* clean lineages were previously genetically engineered to make strains readily usable for laboratory purposes (Cubillos *et al*., 2009). Stable tractable derivatives were thereby developed by disrupting one *HO* allele with the hygromycin resistance cassette and replacing *URA3* with the *KanMX* cassette containing a 6 bp unique 'barcode'. Furthermore, these strains were characterized at both the genomics and the phenomics level (Liti *et al*., 2009; Warringer *et al*., 2011). However, the lack of multiple genetic markers in these new strains has limited their use. In an effort to address this issue and to extend the collection of strains that would be representative of the diversity of the species, we inactivated several of the auxotrophic marker genes commonly used in yeast genetics in the *URA3*- and *HO*-mutated derivative strains. First, *LEU2*, *LYS2* and *MET15* markerless deletions (deletions of entire ORFs without leaving any sequence from positively selectable marker at the corresponding loci) were made by the pop-in/pop-out method (Brachmann *et al*., 1998). Then, the *KanMX* cassette that was originally introduced in *URA3* for deletion (Cubillos *et al*., 2009) was removed by the same technique to allow the recycling of this dominant marker for further constructs. Versions of the *LEU2*, *LYS2* and *MET15* auxotroph strains carrying either *URA3* replaced by the *KanMX* cassette, along with a 6 bp 'barcode' (*ura3Δ::KanMX-Barcode*), or *URA3* deleted without replacement by an additional selectable marker (*ura3Δ0*), were generated. As a result, a collection of 50 strains of *S. cerevisiae* was assembled, including eight haploid and two diploid strains for each lineage, with four auxotroph markers (*LYS2*, *LEU2*, *MET15* and *URA3*) that can be used for further genetic manipulation. This set of strains will undoubtedly be very useful to explore the impact of natural diversity on phenotypic traits and to understand the genetic mechanisms underlying complex traits.

Materials and methods

Strains and media

The original wild-type yeast strains used in this study were previously reported in Liti *et al*. (2009) as part of the *Saccharomyces* Genome Resequencing Project (SGRP). An additional genomic resource was recently released providing *de novo* genome assemblies (http://www.moseslab.csb.utoronto.ca/sgrp/download.html). The five genetic backgrounds

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selected here are representatives of the major diverged lineages described in the SGRP, which were designated Malaysian, West African, North American, Wine/European and Sake. Stable haploid (*MAT***a** or *MATα*, *hoΔ::HphMX*, *ura3Δ::KanMX*) and diploid (*MAT***a**/*MATαHO/hoΔ::HphMX*, *ura3-::KanMX/ura3Δ::KanMX*) derivatives for one representative isolate of each of the five major lineages were subsequently generated and described (Cubillos *et al*., 2009). In this study, these *HO*– and *URA3*-inactivated derivatives were referred to as 'parental strains' (Table 1).

Routinely, yeast cells were grown on yeast extract, peptone and glucose medium (YPD) (Sherman *et al*., 1986). For auxotroph selections, cells were grown on complete synthetic medium (CSM) depleted for the appropriate amino acid (lysine, methionine, leucine) or uracil (Sherman *et al*., 1986). When necessary, YPD was supplemented with hygromycin B 200 μg/ml or geneticin G418 200 μg/ml and CSM was supplemented with 5-fluoro-uracil (5-FoA) 1 g/l. In order to test for petite phenotypes, the 50 auxotroph strains generated in this work were grown on glycerol-containing rich medium.

Transformations of *S. cerevisiae* strains

Transformation of the yeast cells with DNA templates was performed using the lithium acetate method (Gietz and Schiestl, 2007). Briefly, cells were grown in YPD to exponential phase, washed in water, then lithium acetate 0.1 M, and incubated with 1–2 μg DNA (previously linearized pAD plasmid or PCR product) in the presence of carrier DNA (single-stranded salmon sperm DNA) and polyethylene glycol (PEG). The cells were then heat-shocked at 42 °C for 20 min and plated on selective media.

Sporulation and haploid selection

Diploid cells were sporulated for 2–5 days at 30 °C on 2% potassium acetate agar plates. The cells were incubated for 15 min at 37 °C in a 5 mg/ml zymolyase solution. Spores were dissected using a MSM400 Singer (UK) dissection microscope on YPD agar plates and then incubated for 2 days at 30 °C. The colonies were then replica-plated onto appropriate selective media, such as CSM agar plates lacking lysine, leucine, methionine or uracil, in the case of selection for auxotroph mutant strains, or YPD agar plates supplemented with G418 for *ura3Δ::KanMX* strains. Spores from four-viable-spore tetrads with correct marker segregations were selected. Drug resistances and auxotrophies were obtained according to the expected 2:2 segregation. Phenotypes of the spores were checked by restreaking the strains on selective media and amplifying the deleted auxotroph gene loci by PCR.

Crosses between haploid strains and generation of diploids

The mating types of haploid strains were determined by crossing with *MAT***a** and *MATα* tester strains deleted for the *LYS5* gene (*lys5Δ0*). Diploids were selected on minimal medium not supplemented with amino acids or uracil. Growth following crossing with *MAT***a**, *lys5Δ0* indicated that the mating-type of the tested haploid strain

Table 1. Parental *S. cerevisiae* clean lineage strains previously described (Cubillos *et al*.., 2009) and used a starting point for further genetic manipulations

Background	Original strain	Genotype	NCYC Nos
Wine/European	DVBPG6765	(*Mat***a**/*Matα*, *HO*/*hoΔ::HYG*, *ura3Δ::KanMX*/*ura3Δ::KanMX*)	3570 [3597: (MAT**a**), 3622: (MAT*α*)]
West African	DVBPG6044	(*Mat***a**/*Matα*, *HO*/*hoΔ::HYG*, *ura3Δ::KanMX*/*ura3Δ::KanMX*)	3574 [3600: (MAT**a**), 3625: (MAT*α*)]
North American	YPS128	(*Mat***a**/*Matα*, *HO*/*hoΔ::HYG*, *ura3Δ::KanMX*/*ura3Δ::KanMX*)	3581 [3607: (MAT**a**), 3632: (MAT*α*)]
Sake	Y12	(*Mat***a**/*Matα*, *HO*/*hoΔ::HYG*, *ura3Δ::KanMX*/*ura3Δ::KanMX*)	3579 [3605: (MAT**a**), 3630: (MAT*α*)]
Malaysian	UWOPS 03–461.4	(*Mat***a**/*Matα*, *HO*/*hoΔ::HYG*, *ura3Δ::KanMX*/*ura3Δ::KanMX*)	3576 [3602: (MAT**a**), 3627: (MAT*α*)]

NCYC numbers for haploid strains are indicated between square brackets.

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was *MATα* and growth with the *MATα* tester strain characterized a *MAT***a** haploid strain.

To generate diploid strains, two haploid strains with opposite mating types were mixed on YPD agar plates, allowed to grow overnight at 30 °C and restreaked on YPD agar medium to obtain single colonies. In the case of balanced auxotrophies (selection of double- and triple-heterozygous mutant diploids), cells mixed on YPD were replica-plated onto minimal medium lacking all amino acids and supplied with uracil. Diploid colonies were further identified by testing sporulation abilities.

Pop-in/pop-out gene deletions in *S. cerevisiae* 'clean' lineages

UWOPS 03–461.4, DVBPG6044, YPS128, DVBP G6765 and Y12 *URA3*-deleted diploid strains (*ura3Δ::KanMX/ura3Δ::KanMX*) (Table 1) were transformed independently with specific *S. cerevisiae* marker deletion plasmids (ATCC-LGC) for the complete markerless deletion of auxotroph genes based on the pop-in/pop-out deletion method (Brachmann *et al*., 1998). The pAD1, pAD2 and pAD4 plasmids, respectively, carry homologous regions flanking *LEU2*, *LYS2* or *MET15* and specifically target these auxotroph genes for deletion using *URA3* as a selection marker (pAD plasmids carry the *URA3* cassette and confer uracil prototrophy). Plasmid integrants were selected on uracil-depleted CSM. The candidates were confirmed by restreaking on uracil-depleted CSM and integration of the plasmid was checked by PCR at one end of the integration site. The positive clones were heterozygous *LEU2/leu2::pAD1* or *LYS2/lys2::pAD2* or *MET15/met15::pAD4*.

Complete deletions of the targeted auxotroph genes require excision of the inserted pAD plasmids. Diploid transformed colonies were sporulated and dissected to identify four-viable-spore tetrads. Spores were selected for both hygromycin B resistance and uracil prototrophy and grown overnight in YPD liquid medium to be plated onto 5-FoA-containing CSM. After 2 days of growth at 30 °C, 5-FoA-resistant clones, i.e. uracil auxotrophs, were replica-plated onto leucine, lysine or methionine-depleted CSM, with regard to their respective mutation. Auxotrophies were checked on the appropriate corresponding selective media as well as on uracil-depleted CSM. The complete deletion of the targeted gene (and loss of the *URA3*-carrying plasmid) was further confirmed by PCR amplification of the targeted locus. According to this protocol, *leu2Δ0*, *lys2Δ0* or *met15Δ0* single mutants of mating-types *MAT***a** and *MATα* were generated in each of the five *S. cerevisiae* lineages. Triple-mutant strains were obtained by successive rounds of crosses between the single mutant strains and sporulation experiments for the five lineages.

Deletion of *URA3* and removal of the *KanMX* cassette

The *URA3* wild-type gene (1800 bp, containing the ORF and 500 bp both upstream and downstream) was amplified from the genome of the uracil prototroph *S. cerevisiae* tester strain (*lys5Δ0*). This DNA amplicon was used to transform the *LEU2*-, *LYS2*- and *MET15*-deleted mutant strains (haploids *MAT***a**, *hoΔ::HphMX*, *ura3Δ*::*KanMX*, *leu2Δ0*, *lys2Δ0*, *met15Δ0*) in order to restore a wild-type copy of *URA3*. Transformants were selected on uracil-depleted CSM plates. Their uracil prototrophy and G418 sensitivity phenotypes were retested and PCR amplification verified the restoration of the functional *URA3* gene.

The pJL164 plasmid (ATCC-LGC) was used as a template to amplify a deleted version of the *URA3* gene. This piece of DNA includes both upstream and downstream *URA3* ORF flanking regions linked together (without any *URA3* ORF sequence or additional marker). The PCR amplicon was used to transform each uracil prototroph mutant (haploids *MAT***a**, *hoΔ::HphMX*, *leu2Δ0*, *lys2Δ0*, *met15Δ0*) in order to delete *URA3*. Transformants were directly plated on YPD after transformation for overnight growth at 30 °C and replica-plated the next day onto 5-FoA plates. Cells were grown for 1–4 days at 30 °C, depending on the strain. Their uracil auxotrophy phenotype was checked on URA drop-out plates and the deletion of *URA3* was checked by PCR.

Backcross with parental strains

Mutants carrying all four auxotrophies (*MAT***a**, *hoΔ::HphMX*, *leu2Δ0*, *lys2Δ0*, *met15Δ0, ura3Δ0*) were backcrossed twice with their respective parental strain (*MATα*, *hoΔ::HphMX*, *ura3Δ::KanMX*). The first round of backcrossing led to the selection of spores carrying all four auxotrophies, which were further backcrossed once with the parental strains. After the second backcrossing, spores were selected according to the genotypes reported in Table 3.

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Selected haploid auxotroph strains were subsequently crossed to each other to generate diploid strains with balanced gene markers for the *LYS2* and *MET15* alleles (Table 3). All strains are available from the National Culture Yeast Collection (http://www.ncyc.co.uk/) and Accession Nos are listed in Table 3.

Evaluation of cell fitness

Growth assays were performed using Tecan Sunrise 96-well microplate readers and data analysis with Magellan software. First, cells were grown to stationary phase at 30 °C in liquid YPD [optical density (OD) = ~20]. Cultures were then diluted and used to inoculate 100 µl fresh YPD or CSM liquid medium at an estimated initial OD = 0.02. Cultures were grown at 30 °C with constant shaking and ODs were measured every 30 min. Sterile medium was used as control. The parental strains (both *MAT***a** and *MATαhoΔ::HphMX*, *ura3Δ::KanMX* haploids and *HO/hoΔ::HphMX*, *ura3Δ::KanMX/ura3Δ::KanMX* diploid) and the *S. cerevisiae* BY4741 strain were grown along the 50 auxotroph strains in the same conditions. Growth curves were measured in duplicate. Generation times were estimated using a dedicated program (Courbe de Croissance v. 1.4, courtesy of J. Schacherer), which automatically plots (in semi-log scale) the growth curves from the TECAN absorbance data and fits a line to the exponential phase (this fit was manually checked and adapted when necessary). Generation times are automatically calculated from the slope of this line (i.e. the growth rate) as follows: doubling time = ln2/growth rate.

For the Malaysian strains, dry weight experiments were performed in parallel in 10 ml liquid cultures (YPD or CSM). For each strain, four independent cultures were inoculated with either YPD or CSM with the same cell density [100 µl from a small colony (~10^6 cells) resuspended in 1 ml sterile water]. Each of the four cultures was grown under agitation (160 rpm) at 30 °C up to a specific time (four time points from 800 min to 2400 min for the last time point). At the four time points, cells were filtered on 0.22 µm cellulose filters (GSWP04700, Millipore), which were then dried at 65 °C for 2 h and weighed on a scale. Each experiment was performed in duplicate.

Results and discussion

Four auxotrophic marker alleles, *URA3*, *LYS2*, *LEU2* and *MET15*, commonly used in yeast genetics and genomics, were selected for complete markerless deletion. The *URA3*, *LYS2*, *LEU2* and *MET15* genes encode essential enzymes of the uracil, lysine, leucine and methionine biosynthetic pathways, respectively. The four mutations were obtained by pop-in/pop-out (Brachmann *et al*., 1998), using the pAD plasmid series, leading to complete deletion of the targeted protein coding sequences. Precisely, deletion of *LYS2* included the *LYS2* ORF, 304 bp upstream of *LYS2* ATG and 24 bp downstream of its stop codon. Deletion of *LEU2* targeted 6523 bp upstream of ATG and 63 bp downstream of the stop codon; this deletion removed a Ty2 retrotransposon from the DVBPG6765, Y12 and YPS128 strains, while this element is probably absent from the UWOPS 03–461.4 and DVBPG6044 strains, as suggested by SRGP pair-end reads spanning the insertion region (Liti *et al*., 2009). Deletion of *MET15* removed *MET15* ORF, 260 bp upstream of its ATG and 811 bp downstream of the *MET15* stop codon. *URA3* was either replaced from ATG to the stop codon by the *KanMX* cassette (Cubillos *et al*., 2009) or deleted from 222 bp upstream of its ATG to 75 bp downstream of its stop codon. The work flow of the strain construction is illustrated in Fig. 1.

Markerless auxotroph gene deletions in the *S. cerevisiae* 'clean' lineages

One representative isolate of each of the Wine/European, West African, North American, Sake and Malaysian lineages (*ura3Δ::KanMX/ura3Δ::KanMX*) (Table 1), was mutated for *LYS2*, *LEU2* and *MET15*, independently, using the pop-in/pop-out gene deletion method (Fig. 1A). This two-step process includes the integration of a linearized plasmid (pAD) into its specific chromosomal location by homologous recombination (pop-in). Overall, transformations with the integrative plasmids showed good efficiencies in all strains and for all targeted loci (Table 2), averaging 100–1000 transformants/µg pAD1 DNA, 400–1500 transformants for pAD2 and 50–1500 transformants for pAD4. The Malaysian-derived strain, however, yielded 2–30-fold fewer transformants than the other strains. This could possibly be due to the high level of aggregation of this

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strain, which could be detrimental to transformation efficiency (see supporting information, Table S1).

Afterwards, the strains that underwent plasmid excision (pop-out), leading to the deletion of the auxotroph gene, were selected, so that single mutants for either *LEU2, LYS2* or *MET15* were generated in each of the five lineages (Fig. 1A). The observed number of auxotrophic colonies obtained at this step varied greatly, depending on the genetic background and the targeted locus (Table 2). In our hands, the deletion of *LYS2* occurred more frequently than the other two deletions in the five strains (1–30%). *LEU2* and *MET15* deletions highly fluctuated between strains ($< 1\%$ to $>30\%$ and $< 1\%$ to 10%, respectively). However, this result does not seem to correlate with the size of the homology shared between the chromosome and the plasmid, since all homologous regions are similar (~1 kbp). The leucine auxotrophy phenotype was more easily obtained in the Wine/European and North American strains than in the three other strains, whereas mutants for *MET15* were more easily identified in the North American, Sake and Malaysian backgrounds. In the other cases, the efficiencies to obtain leucine and methionine auxotrophs were lower.

Furthermore, the strains did show dissimilar sporulation efficiencies on potassium acetate-containing medium (see supporting information, Table S1). NaCl- and malt-based media were also tested, but did not improve sporulation efficiencies. The North American and Malaysian strains had higher rates of sporulation, which were estimated at 80–90% after 2 days of incubation on potassium acetate medium. The other three lineages required 5–10 days on sporulation media. The West African strain gave usually 10–30% of sporulating cells. The Wine/European and Sake background strains yielded significantly fewer tetrads than the other strains, with 1–5% of sporulation. These results were consistent with the previous report (Cubillos *et al*., 2009).

Subsequently, a *leu2Δ0*, *lys2Δ0*, *met15Δ0* triple mutant in each of the five lineages was generated by successive crossings (Fig. 1B). First, two single mutants with distinct auxotrophies and opposite mating types were crossed to generate a diploid strain. From this diploid strain, spores were dissected to select colonies carrying double auxotrophies. A double-auxotroph haploid strain was then crossed with a haploid strain carrying the third single auxotrophy. Spores obtained from this diploid strain, carrying the leucine, lysine and methionine auxotrophies, were selected (*leu2Δ0*, *lys2Δ0*, *met15Δ0*).

URA3 and *KanMX* cassette as additional selectable markers

The *leu2Δ0*, *lys2Δ0*, *met15Δ0* mutant haploid strains made in each of the five lineages carried a *ura3Δ::KanMX* allele. In order to make complete markerless auxotroph strains which would also allow the recycling of the *KanMX* cassette for further genetic manipulations, we removed the *KanMX* resistance cassette that was used to inactivate the *URA3* gene (Cubillos *et al*., 2009) and generated *ura3Δ0* mutant strains. In each of the five lineages, the *MAT***a**, *ura3Δ::KanMX*, *leu2Δ0*, *lys2Δ0*, *met15Δ0* mutant strain was first transformed with a DNA fragment containing a wild-type copy of the *URA3* gene to restore the uracil prototrophy phenotype (Fig. 1C). The transformation efficiencies varied from one strain to another and reached 38, 5, 48, 189 and 6 transformants/μg DNA amplicon for the Wine/European, West African, North American, Sake and Malaysian strains, respectively (Table 2).

The subsequent uracil prototroph strains were then transformed with a DNA carrying a deleted version of *URA3* corresponding to the entire ORF from the start to the stop codon (Fig. 1C). The estimated transformation efficiency was 6 transformants/μg DNA for the Wine/European, West African and Sake strains, 2 transformants/μg DNA for the North American strain and 1 transformant/μg DNA for the Malaysian strain (Table 2). The quadruple auxotroph strains obtained here were *leu2Δ0*, *lys2Δ0*, *met15Δ0*, *ura3Δ0*.

A set of 50 auxotroph strains to explore *S. cerevisiae* natural variation

In an effort to eliminate putative secondary mutations that might have arisen during the genetic manipulations, the strain carrying all four deletions (*MAT***a**, *leu2Δ0*, *lys2Δ0*, *met15Δ0*, *ura3Δ0*) in each lineage was backcrossed twice with its respective parental strain (*MATα*, *ura3Δ::KanMX*) (Fig. 1D). Spores resulting from these backcrosses and carrying eight different genotypes were collected for each lineage (Fig. 1E, Table 3).

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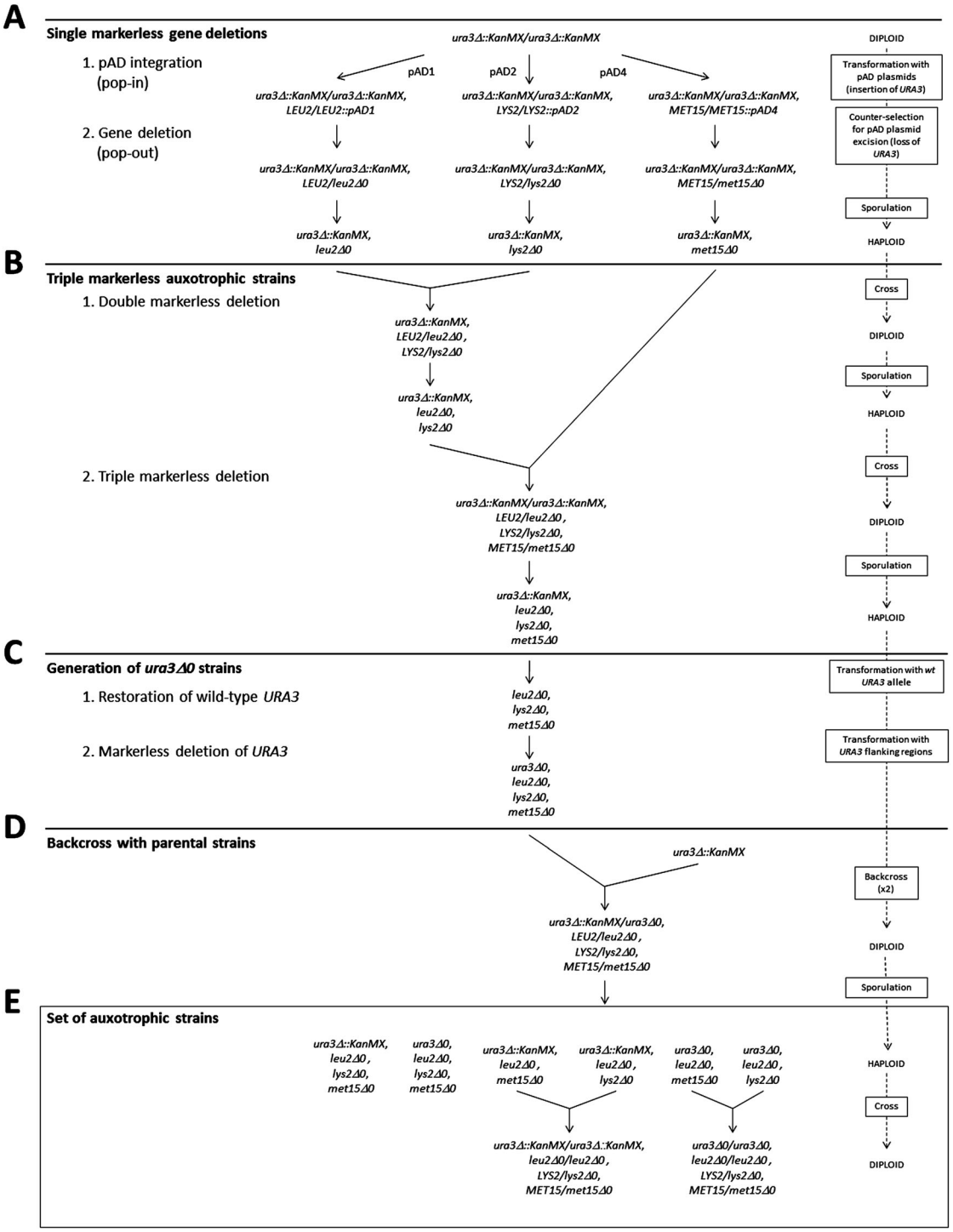

Figure 1. Genealogy of the auxotrophic strains. The successive steps followed in this study are shown. (A) Three auxotrophic genes were independently inactivated in each *S. cerevisiae* lineage without inserting selectable markers (markerless). (B) They were used to make a triple markerless auxotroph mutant strain. (C) A markerless mutated copy of *URA3* (*ura3Δ0*) was made by removing the *KanMX*-barcoded cassette, which was inserted in the *URA3* gene in the parental strains. (D) Backcrossing with the parental strains was realized twice. (E) A set of distinct auxotrophic mutant strains (haploids and diploids) was assembled as indicated

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Table 2. Semi-quantitative data obtained during the genetic manipulation of the strains

Genetic mechanism	*Carrier*	*Targeted gene*	Wine/ European	West African	North American	Sake	Malaysian
Plasmid pop-in[a]	pAD1	*LEU2*	1000	400	200	1000	100
	pAD2	*LYS2*	1500	1400	400	500	100
	pAD4	*MET15*	1500	1400	400	400	50
Plasmid pop-out (%)[b]	pAD1	*LEU2*	> 30	5	< 1	< 1	1.5–4
	pAD2	*LYS2*	1–5	2–30	3	2–10	5–20
	pAD4	*MET15*	7	10	< 1.5	< 1	1–3
Restoration of wild-type *URA3*[a]	PCR product	*URA3*	38	5	48	189	6
Deletion *ura3Δ0*[a]	PCR product	*URA3*	6	6	2	6	1

[a]Number of transformants/μg DNA.
[b]Percentage of auxotrophic cells.

Auxotroph haploid strains for leucine and lysine or leucine and methionine were selected and then crossed to produce two distinct diploid strains with balanced gene markers, i.e. heterozygotes for the *lys2Δ0* and *met15Δ0* mutations (Fig. 1E, Table 3). A collection of 50 strains was assembled, including 40 haploid strains and 10 diploid strains (Table 3). Haploid strains that are mutated for the four biosynthesis pathways of lysine, leucine, methionine and uracil are available in each of the five lineages. These quadruple auxotroph strains will allow the use of most dominant genetic markers, including *KanMX* and *NatMX* (Goldstein and McCusker, 1999), with the exception of the *HphMX* cassette, which was used to delete the *HO* gene, which greatly enhances the possibilities for developing genetic systems. Furthermore, a version of each strain is available with the *ura3Δ0* mutation and the *ura3Δ::KanMX-barcode* mutation labelled with a background-specific barcode, which is designed for identification and quantification purposes. In addition, both *MAT***a** and *MATα* strains were recovered, so that diploid derivatives could be made. The heterozygous diploid strains will also allow the generation of multiple derivatives by combining mutations as needed.

Growth characteristics of the panel of auxotrophic strains

Besides differences in sporulation efficiency (see supporting information, Table S1), the five lineages displayed distinct levels of cell aggregation. For each lineage, we observed under the microscope that cells clump together to form aggregates that cannot be dissociated (either by sonication or by treatment with EDTA or mannose to inhibit lectin-mediated cell aggregation). This was particularly evident in the Malaysian lineage (UWOPS 03–461.4 strain). However, the other backgrounds also showed some levels of aggregation, which were significantly higher for the North American (YPS128) and Wine/European (DVBPG6765) lineages than for the West African (DVBPG6044) and Sake (Y12) strains. These characteristics should be taken into account in experimental designs where cell concentration must be precisely measured.

Further, growth of the 50 auxotroph strains generated in this work was measured in rich and complete synthetic liquid media. Growth curves allowed the calculation of generation times and growth rates for each strain and for their respective parents from the North American, Wine/European, West African and Sake groups (see also supporting information, Figure S1, Table S2). However, the high level of aggregation of the Malaysian strains prevented an accurate measurement of their growth (see below and supporting information, Figure S2).

In our hands, four of the five lineages and their mutant derivatives presented a typical-like growth, so that mutations did not appear to be deleterious to these four lineages. However, it should be noted that the four backgrounds behaved distinctively from each other and that, for instance, their growth curves had different features. In rich medium, the mutant derivatives behaved similarly to their respective parents for all four genetic backgrounds and for both haploid and diploid strains (see supporting information, Figure S1, Table S2). The mutant derivatives also behaved similarly to

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Table 3. Set of 50 auxotroph strains generated in this study

Name	Background	Mating type	*ura3Δ::KanMX*-barcode	*ura3Δ0*	*leu2Δ0*	*lys2Δ0*	*met15Δ0*	NCYC No.
YLF155	Wine/European	**a**	+	–	+	+	+	3882
YLF158	Wine/European	**a**	–	+	+	+	+	3883
YLF156	Wine/European	α	+	–	+	+	+	3884
YLF159	Wine/European	α	–	+	+	+	+	3885
YLF157	Wine/European	**a**	+	–	+	–	+	3886
YLF160	Wine/European	**a**	–	+	+	–	+	3887
YLF154	Wine/European	α	+	–	+	+	–	3888
YLF152	Wine/European	α	–	+	+	+	–	3889
YLF185	Wine/European	**a**/α	–/–	+/+	+/+	+/–	+/–	3890
YLF186	Wine/European	**a**/α	+/+	–/–	+/+	+/–	+/–	3891
YLF183	West African	**a**	+	–	+	+	+	3892
YLF175	West African	**a**	–	+	+	+	+	3893
YLF163	West African	α	+	–	+	+	+	3894
YLF164	West African	α	–	+	+	+	+	3895
YLF184	West African	**a**	+	–	+	–	+	3896
YLF176	West African	**a**	–	+	+	–	+	3897
YLF162	West African	α	+	–	+	+	–	3898
YLF161	West African	α	–	+	+	+	–	3899
YLF187	West African	**a**/α	–/–	+/+	+/+	+/–	+/–	3900
YLF188	West African	**a**/α	+/+	–/–	+/+	+/–	+/–	3901
YLF130	North American	**a**	+	–	+	+	+	3902
YLF131	North American	**a**	–	+	+	+	+	3903
YLF148	North American	α	+	–	+	+	+	3904
YLF149	North American	α	–	+	+	+	+	3905
YLF133	North American	**a**	+	–	+	–	+	3906
YLF132	North American	**a**	–	+	+	–	+	3907
YLF147	North American	α	+	–	+	+	–	3908
YLF146	North American	α	–	+	+	+	–	3909
YLF190	North American	**a**/α	–/–	+/+	+/+	+/–	+/–	3910
YLF189	North American	**a**/α	+/+	–/–	+/+	+/–	+/–	3911
YLF169	Sake	**a**	+	–	+	+	+	3912
YLF170	Sake	**a**	–	+	+	+	+	3913
YLF178	Sake	α	+	–	+	+	+	3914
YLF179	Sake	α	–	+	+	+	+	3915
YLF173	Sake	**a**	+	–	+	–	+	3916
YLF171	Sake	**a**	–	+	+	–	+	3917
YLF177	Sake	α	+	–	+	+	–	3918
YLF181	Sake	α	–	+	+	+	–	3919
YLF191	Sake	**a**/α	–/–	+/+	+/+	+/–	+/–	3920
YLF192	Sake	**a**/α	+/+	–/–	+/+	+/–	+/–	3921
YLF139	Malaysian	**a**	+	–	+	+	+	3922
YLF141	Malaysian	**a**	–	+	+	+	+	3923
YLF140	Malaysian	α	+	–	+	+	+	3924
YLF142	Malaysian	α	–	+	+	+	+	3925
YLF145	Malaysian	**a**	+	–	+	–	+	3926
YLF143	Malaysian	**a**	–	+	+	–	+	3927
YLF138	Malaysian	α	+	–	+	+	–	3928
YLF136	Malaysian	α	–	+	+	+	–	3929
YLF193	Malaysian	**a**/α	–/–	+/+	+/+	+/–	+/–	3930
YLF194	Malaysian	**a**/α	+/+	–/–	+/+	+/–	+/–	3931

+, Deletion present; –, deletion absent.
The *HO* gene was replaced by the *HphMX* cassette in all strains (*hoΔ::HphMX* in haploids and *hoΔ::HphMX/hoΔ::HphMX* in diploids).

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the *S. cerevisiae* BY4741 laboratory strain (see supporting information, Table S2). In complete synthetic medium, the growth of all West African mutant derivatives was similar to that of the parental strain (in both haploid and diploid contexts). However, the growth of the Wine/European, North American and Sake mutant haploid and diploid strains was slower than that of their respective parents, with a 1.5- and 1.8-fold increase of the generation time for the Wine/European haploid and diploid mutants, respectively, and a ~2- and 3-fold increase for the North American and Sake haploid and diploid mutants, respectively. In addition, the generation time of the diploid mutants in minimal medium is significantly higher than that of the corresponding haploid mutant in the Wine/European, North American and Sake groups; being most pronounced in the North American strain (see supporting information, Figure S1, Table S2).

The high level of aggregation of the Malaysian strains prevented the use of the TECAN plate reader to assess fitness. Therefore, dry weight experiments were performed, in both rich and minimal media, to compare growth between mutants and their respective parental strains. For both haploid and diploid mutant strains, no significant growth defect was observed in rich medium, while in minimal medium the mutant strains showed important growth defects relative to their parents (see supporting information, Figure S2).

Altogether, these data emphasize the fact that multiple auxotrophies generally alter cell growth rates in stringent culture conditions, such as when limited nutrients are present (Mulleder *et al.*, 2012). A similar growth defect is also observed for the BY laboratory strains (both haploid and diploid) when cultivated in minimal medium (see supporting information, Table S2). It is nonetheless interesting to note that the West African mutant strains did not show any diminished growth in complete synthetic or rich media.

In conclusion, we envision that the use of this set of markerless auxotroph strains, which originate from highly diverged genetic backgrounds, will allow the development of new approaches to experimentally measure the role of natural variation and genetic interaction upon many biological functions and adaptation processes. Individual genome analysis alone (Jelier *et al.*, 2011), or integrated with additional intermediate phenotypes datasets (Skelly *et al.*, 2013), can be used to develop predictive genotype–phenotype models. It is also interesting to note that a recent multi-locus sequence analysis indicates the presence of eight highly diverged lineages from China (Wang *et al.*, 2012), which vastly expand the known genetic variation of *S. cerevisiae*. An approach similar to the one described in this work could also be applied to these natural isolates (and also to additional lineages described in the future) to broaden our understanding of the relationship between genotypes and phenotypes.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Typical growth curves and generation times measured for the clean lineage parental and mutant strains. Data for one representative haploid mutant strain and one representative diploid mutant strain of the Wine/European, West African, North American, Sake and Malaysian lineages are shown. Growth in rich medium (YPD) or complete synthetic medium (CSM) was followed over 48 h and generation times were calculated. Growth curves for one representative haploid mutant strain (mata) of the Wine/European, West African, North American, Sake and Malaysian lineages, YLF155, YLF183, YLF130, YLF169 and YLF139 (see Table 3), respectively, are presented. Growth curves obtained from haploid or diploid mutant strains and from their respective parental strains were similar. Generation times for the haploid parental (white bar) and mutant (hatched bar) strains as well as for the diploid parental (grey bar) and mutant (grey bar with squares) are presented, except for the Malaysian lineage, because of its high level of aggregation. The error bars correspond to standard error of the mean. Representative diploid mutant strain of the Wine/European, West African, North American, and Sake lineages were YLF185, YLF187, YLF190 and YLF191 (see Table 3), respectively

Figure S2. Dry weight-based growth curves of Malaysian strains. Growth curves were obtained for haploid and diploid parental strains (UWOPS 03-461.4) as well as for one representative haploid and diploid mutant strain (YLF139 and YLF193, respectively; see Table 3). Cells were cultivated at 30°C under agitation in rich (YPD) or minimum (CSM) medium for about 40 h (2400 min) and their weight was recorded. Each experiment was performed in duplicate. The error bars correspond to the standard error of the mean.

Table S1. Estimated level of aggregation and sporulation efficiency for each clean lineage.

Table S2. Estimated generation times (G) and growth rates (μ) of parental strains and auxotrophic strains generated in this study

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