Designer Deletion Strains derived from *Saccharomyces cerevisiae* S288C: a Useful set of Strains and Plasmids for PCR-mediated Gene Disruption and Other Applications

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A set of yeast strains based on *Saccharomyces cerevisiae* S288C in which commonly used selectable marker genes are deleted by design based on the yeast genome sequence has been constructed and analysed. These strains minimize or eliminate the homology to the corresponding marker genes in commonly used vectors without significantly affecting adjacent gene expression. Because the homology between commonly used auxotrophic marker gene segments and genomic sequences has been largely or completely abolished, these strains will also reduce plasmid integration events which can interfere with a wide variety of molecular genetic applications. We also report the construction of new members of the pRS400 series of vectors, containing the *kanMX*, *ADE2* and *MET15* genes. © 1998 John Wiley & Sons, Ltd.


KEY WORDS — *Saccharomyces cerevisiae*; yeast; gene disruption; S288C; bacteria-yeast shuttle vectors; auxotrophic markers

INTRODUCTION

Deletion mutations are valuable because they never revert. This is particularly true with regard to DNA transformation because there is no background of revertants to sort through; any organism that acquires the wild-type phenotype is by definition a transformant. A problem with most deletion alleles is that they rarely precisely affect a single gene; there may be 'collateral' effects on one or more adjacent genes that can confound genetic analysis. The availability of whole-genome sequence information together with modern recombinant technologies like the polymerase chain reaction (PCR) allows the precise design of deletions that are much less likely to have such problems. We have designed a strategy exploiting these technologies to create a set of precise deletion alleles of commonly used auxotrophic markers in the yeast *Saccharomyces cerevisiae*. As additional complete genomes are sequenced, this strategy can be used to create high quality deletion alleles in other organisms.

Traditionally the yeast *S. cerevisiae* has served as one of the best genetically tractable eukaryotic systems. Researchers have for years been able to disrupt genes in yeast in order to study their mutant phenotypes; however, the techniques used for gene disruption have had limitations. First, the gene to be disrupted had to be cloned. Second, because available restriction sites were used to delete a portion of the gene and insert a selectable marker, 5' or 3' fragments of the gene were often

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left in the genome, raising potential doubt about the null phenotype. A method has recently been developed that allows complete deletion of the gene of interest and its replacement with an auxotrophic marker gene (Baudin et al., 1993; Lorenz et al., 1995). This procedure, called PCR-mediated gene disruption, requires only knowledge of the genomic sequence of the gene of interest, allowing for precise gene deletion prior to its cloning and immediate study of the null phenotype. Along with the availability of the entire S. cerevisiae genomic sequence, the method of PCR-mediated gene disruption has changed the face of yeast genetics, enabling researchers to quickly assess a gene’s possible function through evaluation of the null phenotype, and to efficiently tackle the more complex problems inherent in the analysis of multi-gene families.

Like its predecessor method, one-step gene disruption (Rothstein, 1983), PCR-mediated gene disruption relies on the fact that homologous recombination in yeast can be efficiently mediated by linear fragments of DNA. The method requires two ~60 nucleotide (nt) PCR primers; the 20 nts of sequence at the 3’ ends of each primer is specific for the amplification of an auxotrophic marker gene, and the 40 nts of sequence at the 5’ ends is identical to the left and right genomic sequence flanking the gene of interest. Using these targeting primers, the auxotrophic marker gene is amplified and the resulting PCR product contains the marker gene and 40 bp of genomic DNA sequence to the left and right of the gene deletion of interest. This product is then transformed into yeast, and stable transformants are selected on appropriate medium. These transformants include those cells in which two crossover events occurred, one between each end of the 40 bp target sequences of the PCR product and the corresponding genomic DNA sequence, thereby replacing the gene of interest with the auxotrophic marker gene.

A limitation of this method has been that the genomic alleles of many of the most commonly used auxotrophic markers are point mutations, small internal deletions, or Ty1 insertions, and therefore retain large regions of homology to the internal selectable marker segment of the PCR product. This results in a high background of positive transformants in which the marker gene has replaced the auxotrophic mutation by marker locus gene conversion, leaving the gene of interest unaffected. This problem is circumvented by use of the set of ‘designer’ deletion strains we have made, in which some commonly used auxotrophic marker genes have been completely deleted. These strains eliminate the background of undesired marker gene convertants. Because the homology between commonly used auxotrophic marker gene segments and genomic sequences has been largely or completely abolished, these strains will also reduce plasmid integration events which can interfere with a wide variety of molecular genetic applications. We have also constructed a designer deletion allele of the MET15 gene, a selectable marker gene which is useful both because it is counterselectable and because it can be used as a colony color marker (Cost and Boeke, 1996).

Additionally, we have increased the repertoire of the pRS plasmids by constructing new versions containing the kanMX marker (Wach et al., 1994), ADE2 and MET15 markers. Finally, we have designed universal PCR primers for PCR-mediated gene disruption. This strategy allows the amplification of each of the auxotrophic marker genes found in the pRS set of plasmids (Sikorski and Hieter, 1989). Thus, the same primers can be used to amplify any of the auxotrophic marker genes, allowing for much greater flexibility in the design of gene knockout experiments.

MATERIALS AND METHODS

Strains and media

The yeast strains are all directly descended from FY2 (Winston et al., 1995), which is itself a direct descendant of S288C, and are described in Table 1. The genealogy of the strains in this paper is summarized in Figure 1. Media used were as described (Boeke et al., 1984; Rose et al., 1990), or as described in Table 2 for scoring the met15 marker. Efficient sporulation of diploids required growth as a patch on freshly poured GNA pre-sporulation plates (5% glucose, 3% Difco nutrient broth, 1% Difco yeast extract, 2% agar) for 1 day at 30°C prior to transfer to liquid sporulation medium (1% potassium acetate, 0.005% zinc acetate). Typically, a generous matchhead of cells was resuspended in 2 ml of liquid sporulation medium. Sporulation cultures were incubated on a roller wheel for 1 day at 25°C followed by 3–5 days at 30°C and could be readily dissected. In general, sporulation efficiencies were of the order of 20–30% (pre-growth of diploids on YPD instead of GNA resulted in somewhat lower sporulation efficiencies).
Table 1. Yeast strains.

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Data in this table are maintained electronically at http://www.welch.jhu.edu/~gregory/MET15.html.

a ATCC, American Type Culture Collection, Rockville, MD.

b Strains selected as parent strains for the international systematic Saccharomyces cerevisiae gene disruption project.

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Of marker flanking sequences

Oligos LEU2-1 (cgggttaccACAGAGTACTT ATACGTAC—small letters indicate restriction sites added) and LEU2-2 (cgggttaccGAGAACA TTCATGATTAGAGG) were used to amplify a 1010 bp region to the left of LEU2 and oligos LEU2-3 (cgggttaccCGACACAGAAATTACAAA ATG) and LEU2-4 (cggctggagtcCAAGTGTTGTC TTGGAAGCCG) were used to amplify a 1220 bp region to the right of LEU2. Oligos LYS2-1 (cgggttaccGTACCTTTTGAACTTGTC) and LYS2-2 (cgggttaccGAAGCGTCAGGAAGA AG AAA) were used to amplify a 917 bp region to the left of LYS2 and oligos LYS2-3 (cgggttacTTCATGATAATTTATATGAATTAGG) and LYS2-4 (cggctggagtcCATCATGCTGCAGA
Table 2. Medium used for scoring met15.

<table>
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<td>3 g peptone</td>
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<tr>
<td>5 g yeast extract</td>
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</tr>
<tr>
<td>200 mg ammonium sulfate</td>
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</tr>
<tr>
<td>20 g agar</td>
<td></td>
</tr>
<tr>
<td>q.s. 800 ml with water</td>
<td></td>
</tr>
<tr>
<td>Autoclave</td>
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</tr>
<tr>
<td>1 g lead nitrate</td>
<td>(add 2 ml 0.5 g/ml filter-sterilized stock)</td>
</tr>
<tr>
<td>40 g glucose</td>
<td>(add 200 ml 20% stock)</td>
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*Lead plates are useful because met15 strains are dark brown to black, whereas wild-type strains are white. To score the Met− auxotrophic phenotype, use SD plates supplemented with the requirements of the strain. Note that SC− met medium cannot be used unless cysteine is also omitted, as either methionine or cysteine allows growth of met15 mutants (Ono et al., 1991; Cost and Boeke, 1996).*

Table 3. Description of Δ0 deleter plasmids.

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<th>Enzyme for linearization</th>
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<td>SpeI, XhoI</td>
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*Other cloned deletions*

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<td>99604</td>
<td>ade2::hisG::URA3::hisG</td>
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</table>

*The set of four designer deletion plasmids in this table is available from ATCC as a kit (87472) of four strains.

*Cutting with the indicated enzymes will target the plasmid to the desired locus. In the case of pJL164, two enzymes are used to release the fragment containing the deleted genomic region of URA3. Deletion of URA3 requires two homologous recombination events.

*Two-step gene replacements (indicated as Ura+, FoaR) are required for most of these plasmids. For pJL164, which is a single-step replacement, cells are outgrown on YPD at 30°C prior to replica-plating to Foa medium. See Materials and Methods for details.*

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construction, all 5′ overhangs created by restriction digests were blunted by filling in with the Klenow large fragment of *Escherichia coli* DNA polymerase, and all 3′ overhangs were made blunt using the 3′ to 5′ exonuclease activity of T4 DNA polymerase. All sites indicated on the restriction maps were verified by digestion.

**Construction of the MET15 plasmids, pRS401, pRS411 and pRS421**  The pRS40X series precursor plasmid pJK142 (Keeney and Boeke, 1994) was digested with *NdeI*, blunted and the minimal complementing 1621 bp *EheI*–*ScaI* fragment of the MET15 genomic locus was inserted to create pRS401 (*EheI* recognizes the same site as *NarI* but leaves a blunt end). pRS401 was digested with *AatII*, blunted, and a 514 bp *HinClI*–*PstI* (blunt) fragment of pRSS84 containing the *CEN6/ARS84* cassette (Sikorski and Hieter, 1989) inserted to create pRS411. pRS401 was digested with *AatII*, blunted, and the 1345 bp *HpaI*–*NdeI* (blunt) fragment of YEpl2 containing the 2 μ ori (A form; Christianson *et al*., 1992) was inserted to create pRS421.

**Construction of the ADE2 plasmids, pRS402, pRS412 and pRS422**  pJK142 was digested with *NdeI*, blunted, and a 2252 bp *BglII* (blunted) fragment of pASZ11 (kindly provided by Patrick Linder) containing a minimal complementing region of the ADE2 genomic locus (Stotz and Linder, 1990) was inserted to create pRS402. The *CEN6/ARS84* plasmid pRS412 and the 2 μ plasmid pRS422 were created by ligation of the 1246 bp *ApaII* fragment of pRS402 and the 3781 bp *ApaII* fragment of pRS402 with either the 1011 bp *CEN6/ARS84* *ApaII* fragment of pRS411 or the 1837 bp 2 μ *ApaII* fragment of pRS421. It should be noted that these ADE2 clones bear a silent A to G mutation at nucleotide 1243 of the GenBank sequence designed to destroy a *BglII* restriction site (Stotz and Linder, 1990).

**Construction of the kanMX4 pRS400 plasmid**  pJK142 was digested with *NdeI*, blunted, and a 1483 bp *SmaI* to *EcoRV* fragment of pFA6A (Wach *et al*., 1994) containing *kanMX4* was inserted, and kanamycin-resistant transformants were isolated. The initial clone was found to contain two copies of the vector sequence ligated in tandem with a single copy of *kanMX4*. This clone was digested with *AatII* and re-ligated to create pRS400 which contains a single copy of the vector and *kanMX4*.

**Construction of markerless pRS vectors**  The *CEN6/ARS84* plasmid pGC25 and the 2 μ plasmid pGC26 were created by ligation of the 1246 bp *ApaII* fragment of pRS402 and the 1527 bp *ApaII* fragment of pJK142 with either the 1011 bp *CEN6/ARS84* *ApaII* fragment of pRS411 or the 1837 bp 2 μ *ApaII* fragment of pRS421. These plasmids contain either the *CEN6/ARSH4* or the 2 μ sequence, but lack a selectable marker for use in *Saccharomyces* *cerevisiae*. pGC25 (*CEN*) and pGC26 (2 μ) should be useful as the basis for a further extension of the pRS line to encompass an even greater diversity of markers.

**Introduction of gene disruption mutations into yeast and strain history**

We introduced the ade2Δ::*hisG* mutation into YPH681 (a direct descendant of FY2) to create YCB436 using the previously described ADE2 disrupter pAADE2 (Aparicio *et al*., 1991), which results in deletion of the ADE2 ORF (with the exception of six C-terminal amino acids) and replacement with a copy of the bacterial *hisG* gene. To create the correct starting strains for the designer deletions, YCB436 was crossed to BY378 and the resulting diploid was sporulated. Two spores from this cross, YCB451 and YCB452 were then gene converted to *URA3* by transformation with the 13 kb *EcoRI* fragment containing *URA3* (derived from pSK179 provided by S. Kunes) and selection for *Ura*+, thus creating strains BY397 and BY398, respectively. To introduce the ura3ΔM0 deletion, the *SpeI*–*XhoI* fragment from pJL164 was transformed into these two strains, transformants were grown for 1 day at 30°C on YPD plates to allow loss of pre-existing Ura3 protein and development of the FOA™ phenotype (Ronne and Rothstein, 1988) and then replica-plate to 5-FOA plates. 5-FOA™ colonies were identified and patched onto YPD. These were screened for *URA3* deletions by a reversion analysis. The YPD plates were replica-plate to SC – ura plates and irradiated with UV-light at 100 J/m² using a Stratagene (Stratagene; setting of 100 μJ × 100/cm²). Following irradiation, plates were wrapped in foil to prevent photoreactivation and incubated for several days at 30°C. Using this test, *ura3ΔM0* deletions were identified by their inability to revert to
Ura⁺, whereas point mutations reverted. Finally, correct ura3Δ0 deletions in strains BY399 and BY401 were confirmed by Southern blot analysis (Ausubel et al., 1987).

Figure 2 describes the method used to introduce the remaining designer deletion mutations. First, deleter plasmids pAD1, pAD2 and pAD4 were linearized at unique sites within each right flanking fragment (pAD1 with SaII, pAD2 and pAD4 with CclI) and transformed into the ura3Δ0 strains. Genomic integrants were selected on SC–ura plates. Ura⁺ colonies were replica-plated to YPD plates to allow recombinational loss of the integrated URA3 marker and pRS406 backbone and then replica-plated to 5-FOA plates to identify those that had become Ura−. 5-FOA-resistant colonies were picked and checked for loss of the targeted auxotrophic marker by replica-plating to SC–leu, SC–lys or lead-containing plates (mer15 strains are brown on these plates). Finally, deletions were confirmed by Southern analysis. The pAD1 transformation was performed in strain BY399 to create BY404, the pAD2 transformation was performed in strain BY401 to create BY406 and the pAD4 transformation was performed in strain BY406 to create BY411.

To create a set of strains consisting of a varying array of deletion alleles, BY411 (MATα ura3Δ met15Δ0 trp1Δ63 his3Δ200 ade2Δ lys2Δ0) was crossed to BY379 (MATα his3Δ200) and a spore was selected with the following genotype: MATα met15Δ0 his3Δ200 lys2Δ0. This strain, BY413, was mated to BY404 (MATα ura3Δ met15Δ0 trp1Δ63 his3Δ200 ade2Δ). Tetrad dissection of four resulting diploids yielded an unexpected result: two spores of each tetrad grew slowly on rich medium, irrespective of genotype. Suspecting that these might have resulted from a single nuclear petite mutation, we tested all tetrads for growth on glycerol- and ethanol-containing rich medium (petite strains cannot utilize these carbon sources and so fail to grow). We found that these slow-growing spores did not exhibit a petite phenotype since all spores grew on these plates. We backcrossed one of the faster-growing spores from this cross (BY418; MATα ura3Δ met15Δ0 trp1Δ63 his3Δ200 ade2Δ lys2Δ0 leu2Δ0) to the parent FY4 (MATα) prototrophic strain. All spores obtained from tetrad dissection of this cross grew equally well, indicating that the mutation causing slow growth observed in the previous cross had been eliminated. The strain history is presented schematically in Figure 1).

**Northern analysis of flanking genes**

RNA was prepared from 10 ml of a log-phase culture (OD₆₀₀=1–2) grown in YPD at 30°C. The cells were washed once in water and resuspended in 0.3 ml RNA buffer (0.1 M NaCl, 0.1 M Tris base, 0.03 M EDTA, 1% w/v N-lauryl sarcosine, pH 8.9–9.0) and frozen at −70°C. Upon thawing, 0.4 g glass beads, 0.15 ml phenol and 0.15 ml chloroform were added, and the cells were broken by vortexing for 10 min. Following a short spin to separate the phases, 1/10th volume 3 M NaOAc and 2–5 volumes ice-cold EtOH were added to the aqueous layer to precipitate the nucleic acids (mostly RNA). The precipitate was dissolved in 100 μl of loading buffer (50% formamide, 25% water, 15% formaldehyde, 10% 10× MOPS–0.2 M morpholinopropane-sulfonic acid, 0.05 M NaOAc, 0.01 M Na₂EDTA; pH adjusted to 7.0 with NaOH). 10 μg RNA was first heated to 55°C for 15 min and then electrophoresed on a 1%-2% agarose/formaldehyde gel as described (Ausubel et al., 1987). The separated RNA was transferred to Genescreen Plus (ICN) and hybridized to the appropriate probe (Ausubel et al., 1987) and Genescreen Plus protocol.

**PCR protocol for PCR-mediated gene disruptions**

Using primers consisting of 40nts of gene-specific sequence at the 5’ end followed by: 5’-CTGTGCGGTATTTTACACCG-3’ (left primer) and 5’-AGATGGACTGAGAGTGCACT-3’ (right primer), any auxotrophic marker can be amplified from a pRS40X integrating plasmid using the following PCR protocol: 94°C 2 min, [94°C 1 min, 55°C 1 min, 72°C 2 min] × 10 cycles, [94°C 1 min, 65°C 1 min, 72°C 2 min] × 20 cycles, 72°C 10 min. This protocol usually results in amplification of only the specific product. If yields are high enough, it is possible to directly transform yeast, but generally, 10-fold concentration by EtOH precipitation before transformation is desirable.

**RESULTS AND DISCUSSION**

A compilation of commonly found alleles of auxotrophic marker genes

To develop a strategy for construction of designer deletion strains, we first had to compile relevant information for existing auxotrophic selectable marker alleles. Many commonly used strains contain mutations in the auxotrophic
Table 4. A compilation of commonly used auxotrophic marker mutations.

<table>
<thead>
<tr>
<th>Allele</th>
<th>ΔORF?</th>
<th>Reverts?</th>
<th>Notes</th>
<th>Molecular description$^a$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ade2-101</td>
<td>No</td>
<td>Yes</td>
<td>Ochre mutation, red colonies</td>
<td>Frameshift (BglII site filled in at pos. 592)</td>
<td>Engebrecht and Roeder (1990)</td>
</tr>
<tr>
<td>ade2-BglII</td>
<td>No</td>
<td>No</td>
<td>Red colonies</td>
<td></td>
<td>Struhl (1985); Fasullo and Davis (1988); Siram et al. YGM RNA processing mtg (1993)</td>
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<tr>
<td>his3Δ200</td>
<td>Yes</td>
<td>No</td>
<td>Cold sensitive; high frequency of petite formation, especially during transformation</td>
<td>Δ 1 kb (−205–835)</td>
<td></td>
</tr>
<tr>
<td>his3Δ1</td>
<td>Partial</td>
<td>No</td>
<td></td>
<td>Δ 187 bp HindIII–HindIII internal (305–492)</td>
<td>Scherer and Davis (1979)</td>
</tr>
<tr>
<td>his3-11,15</td>
<td>No</td>
<td>No</td>
<td>Double mutant</td>
<td></td>
<td>Lau and G. R. Fink, unpublished</td>
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<tr>
<td>leu2ΔI</td>
<td>Partial</td>
<td>No</td>
<td></td>
<td>Δ 0.6 kb, EcoRI–ClaI internal (163–649)</td>
<td>Sikorski and Hieter (1989)</td>
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<tr>
<td>leu2-3,112</td>
<td>No</td>
<td>No</td>
<td>Double mutant</td>
<td>leu2-3 is a +1 frameshift mutation</td>
<td>Hinnen et al. (1978); Gaber and Kulbertson (1982)</td>
</tr>
<tr>
<td>lys2-801</td>
<td>No</td>
<td>Yes</td>
<td>Amber mutation</td>
<td>Δ 1·0 kb, XhoI–HpaI internal (1813–2864)</td>
<td>Winston et al. (1995)</td>
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<tr>
<td>lys2Δ202</td>
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<td>No</td>
<td></td>
<td></td>
<td>Sikorski and Hieter (1989)</td>
</tr>
<tr>
<td>trp1ΔI</td>
<td>Yes</td>
<td>No</td>
<td>Cold sensitive$^b$, weak galactose inducer (deletes GAL3 UAS), removes ARS1, also called trp1-901</td>
<td>Δ 1·45 kb, EcoRI–EcoRI (−102 to 1352)</td>
<td></td>
</tr>
<tr>
<td>trp1Δ63</td>
<td>Partial</td>
<td>No</td>
<td>Cold sensitive$^b$</td>
<td>Δ 0·6 kb, EcoRI–HindIII (−102 to 513)</td>
<td>Sikorski and Hieter (1989)</td>
</tr>
<tr>
<td>trp1-289</td>
<td>No</td>
<td>Yes</td>
<td>Cold sensitive$^b$</td>
<td>Tyl insertion (transcribing left to right) at pos. 121</td>
<td>Rose and Winston (1984)</td>
</tr>
<tr>
<td>ura3-52</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
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<td>ura3-1</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ade2Δ::hisG</td>
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<td>No</td>
<td>Designer deletion</td>
<td></td>
<td>Aparicio et al. (1991)</td>
</tr>
<tr>
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<td>No</td>
<td>Designer deletion</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>lys2Δ0</td>
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<td>No</td>
<td>Designer deletion</td>
<td>This study</td>
<td></td>
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<tr>
<td>met15Δ0</td>
<td>Yes</td>
<td>No</td>
<td>Designer deletion</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>ura3Δ0</td>
<td>Yes</td>
<td>No</td>
<td>Designer deletion</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

An updatable version of this table is maintained by SGD (http://genome-www.stanford.edu/Saccharomyces/).

$^a$The sequence coordinates are relative to the first ATG of the selectable marker ORF, in which the A residue is defined as +1.

$^b$All Trp$^−$ strains are cold sensitive (Singh and Manney, 1974).

marker genes—namely, point mutations or small internal deletions which are not conducive to successful PCR-mediated gene disruption. These auxotrophic loci retain extensive homology with the prototrophic marker gene segment of the PCR product, leading to high levels of mistargeting. We have compiled a table of many of these commonly used alleles (Table 4) which should be useful in determining a strain’s adaptability for PCR-mediated gene disruption.
PCR-mediated gene disruption is variably successful in its degree of targeting the locus of interest, even when complete deletion alleles corresponding to the marker gene being introduced are used. The reason for this variability in efficiency is not entirely clear but appears to be related primarily to the targeting sequences that flank the target gene (Q. Feng and J.D.B., unpublished data), and to a lesser extent to the nature of the disrupting auxotrophic marker. It is important to note that the commonly used segment of LEU2 (the XhoI-SalI fragment found in the pRS vectors) contains 240 bp of δ (Ty2 LTR) sequence at its 5’ end. This may lead to a higher frequency of mistargeting than with the other auxotrophic markers.

**Complete deletion alleles of URA3, LEU2, LYS2 and MET15**

Commonly used strains of the S288C lineage contain only four auxotrophic alleles that are well-defined deletions, namely his3Δ200, leu2Δ1, trplΔ1, and trplΔ63. Both the his3Δ200 and trplΔ1 mutations completely remove the protein coding regions, whereas trplΔ63 leaves intact the 3’ end of the gene and leu2Δ1 is an internal deletion (Table 4). All of these mutations except leu2Δ1 work well for the method of PCR-mediated gene disruption, resulting in proper gene disruptions 30–100% of the time. Thus, new designer deletion alleles of TRP1 and HIS3 have not been constructed. However, PCR-mediated gene disruptions using other commonly used auxotrophic alleles have been difficult to obtain because of the high background of gene conversion. For this reason we set out to construct an isogenic set of strains in which URA3, LYS2, LEU2 and MET15 were completely deleted.

The strategy for the designer deletion alleles (designated Δ0) is depicted in Figure 2. Approximately 1 kb ‘left’ and ‘right’ flanking regions of the auxotrophic marker to be deleted were cloned into a URA3 integrating vector. Linearization of the resulting deletion plasmid within the right flanking sequence and transformation of a ura3Δ0 strain results in integration of the plasmid at the auxotrophic marker locus. Subsequent recombination between the duplicated left or right flanks results in 5-FOA resistance (due to excision of the URA3 plasmid) and leads to acquisition of the appropriate auxotrophy (in principle, 50% of the time).

Because this strategy involves integration of a URA3 containing plasmid, it was important that our starting strain was not only Ur -, but also lacked sequences overlapping with the plasmid URA3 gene. To completely delete URA3, the plasmid pJL164, containing a genomic 4.0 kb BglII–BamHI fragment in which the region from HindIII to SmaI encompassing the URA3 gene has been deleted (Figure 3), was digested with SpeI and XhoI to liberate a DNA fragment spanning the deletion (Table 3). This was transformed into the URA3+ strains BY397 and BY398 and Ur - colonies were selected on 5-FOA. Reversion tests and Southern blotting confirmed the acquisition of the ura3Δ0 allele in strains BY399 and BY401.

All four designer deletion plasmids completely remove the open reading frame encoding the appropriate auxotrophic marker. They also remove several hundred bp of 5’ flanking region, and as much 3’ flanking region as is practical. Ideally, there should be no homology between the marker segment in the plasmid to be used and the deletion in the chromosome. In two cases (ura3 and met15) it was possible to remove all homology between plasmid marker segment and chromosomal deletion. The details of the design of each deletion were dictated by what was known about the nature of flanking genes; often, as at the 3’ end of LYS2, there was not much room to maneuver due to the proximity of the adjacent gene (Figure 3). Both MET15 and LEU2 are flanked by tRNA genes and δ elements and in the case of LEU2, an entire Ty2 element. Because the segment of LEU2 commonly used in cloning vectors contains part of this Ty2 element as well as a tRNAe-leu gene, it was necessary to delete the Ty2 element, the tRNA gene, and LEU2 in order to eliminate overlap with the vector segment, at least at one end of the marker gene. Thus the leu2Δ0 allele is considerably larger than the rest. No unusual phenotypes have resulted from this deletion.

To create the leu2Δ0 and lys2Δ0 strains, pAD1 was linearized with SalI and pAD2 with ClaI (Table 3 and Figure 2) and transformed into BY399 and BY401 respectively. Ur+ colonies were patched onto SC–ura, replica-plated to YPD (to allow loss of the URA3+ and flanking vector sequences) and replica-plated to 5-FOA plates. Papillae from the 5-FOA plates were streaked onto YPD and replica-plated to either SC–leu or SC–lys to identify auxotrophs (Figure 2). Of 24 5-FOA R papillae from the pAD1
transformation, three were Leu−, and of 24 5-FOA R papillae from the pAD2 transformation, six were Lys−. The deletions were confirmed by genomic Southern analysis (Figure 4).

The met15Δ0 mutation was introduced into BY406 (the lys2Δ0 derivative) in a similar fashion. Plasmid pAD4 was linearized with ClaI and transformed. Following 5-FOA selection, candidate strains were streaked onto YPD plates and replica-plated to lead-containing plates. When grown on these plates met15 strains are brown in color, identifying those strains in which recombinational loss of the URA3 + vector sequences had resulted in a Met− phenotype. The met15Δ0 allele was confirmed by Southern analysis (Figure 4).

Using the ADE2 disrupter plasmid pΔADE2 (Aparicio et al., 1991, the ade2Δ::hisG::URA3::hisG (originally referred to as ade2Δ by Aparicio et al.) mutation was introduced into these strains; these were converted to ade2Δ::hisG alleles by plating on 5-FOA as above. Since six C-terminal
amino acids remain of the ADE2 ORF, the ade2Δ:hisG mutation is not technically a designer deletion, and it has been shown to be useful in PCR-mediated gene disruption (D. Gottschling, pers. comm.).

Because these strains are intended to be used for, among other things, the systematic genetic analysis of genes of unknown function, it was important to establish that expression of the ORFs neighboring the deleted auxotrophic markers was unaffected in these deletion strains. This seemed especially important because many yeast workers have found that two of the commonly used deletion alleles, his3Δ200 and trp1Δ1, have ‘collateral’ phenotypes that are sometimes undesirable due to effects on adjacent genes. his3Δ200 strains display an increased rate of petite formation, presumably due to effects on expression of the neighboring PET56 gene. trp1Δ1 removes ARS1 (with no obvious phenotype) and partially disables the neighboring GAL3 gene by removing a portion of its UAS. Finally, both trp1Δ63 and trp1Δ1 strains share a cold-sensitivity phenotype which all Trp− strains exhibit (Singh and Manney, 1974).

It is impossible to totally rule out effects on flanking genes but the deletions were designed to minimize the possibility of such effects. In the construction of the leu2 and met15 deletions, tRNA genes that are part of multigene families were removed in addition to the auxotrophic marker. The phenotypic consequence of this is likely to be minimal, based on experiences with other multi-gene tRNA families (Byström and Fink, 1989; Åström and Byström, 1993). All of the designer deletions remove marker genes that are flanked by convergently transcribed neighboring genes (Figure 3); effects on the flanking genes are thus expected to be minimal. Nevertheless, the possibility of altered transcript sizes and transcriptional level of neighboring genes was investigated by Northern analysis. RNA was prepared from BY418 (which has all four designer deletion mutations) and FY2 (the wild-type strain), and left and right flanking probes (Figure 3) were used to detect the transcription of neighboring ORFs; in no case was the expression of a neighboring ORF significantly affected (Figure 5; compare the neighboring gene specific transcript with the Ty1 loading control transcript). However, our analyses did detect one minor but interesting expression pattern change. The LEU2 neighboring ORF, YCL17C (reading toward LEU2), appears to direct a major transcript and numerous larger minor transcripts.

In the leu2Δ0 strain, the overall levels of the transcripts are slightly reduced and in particular, the largest transcripts appear to be missing. Such an observation is consistent with the possibility that the larger transcripts normally terminate within the deleted region.

A set of designer deletion strains

To obtain strains with numerous different combinations of auxotrophic alleles, 206 tetrads were dissected from a cross between FY3 and BY418 (Figure 1). This cross gave ~30% sporulation and 96-5% spore viability using the conditions described in Materials and Methods. A subset of the strains from this cross identified in Table 1, which should prove generally useful to the yeast community, has been deposited with the ATCC.

New pRS plasmids with ADE2, MET15 and kanMX4 markers

The pRS vector series is a set of S. cerevisiae shuttle plasmids that are in wide use. Much of their appeal derives from their small size, modular design, minimal length marker segments, useful polylinker and consistency of structure. Also, complete DNA sequence files exist for these plasmids, greatly facilitating construction and analysis of recombinant plasmids. The original set of pRS vectors (the pRS300 series) were based on pBluescript KS+ (Sikorski and Hieter, 1989) and included both integrating and centromere-containing versions; the pRS400 series is based on pBSII SK+ (Stragartone; Christianson et al., 1992) and also includes 2 μ versions. This latter series is more generally useful for several reasons: (1) the lacZ a-complementation is improved over the pRS300 series; (2) the polylinker is flanked by BssHII sites, allowing a simple assessment of insert size to be carried out and facilitating swapping of inserts from one vector to another; and (3) the pRS400 series is more complete, including integrating, CEN, and high copy versions. The ‘code’ for the pRS vectors is simple: the second digit (0, 1 or 2) specifies the plasmid type (integrating, CEN or 2 μ, respectively) and the third digit specifies the selectable marker (see Table 5).

We constructed new integrating, CEN and 2 μ plasmids for use with the ade2Δ and met15Δ0 strains (Figure 6). In all cases, the plasmid backbone is identical to the pRS400 series of plasmids, and the selectable markers were inserted into the same restriction site as had been utilized for
the pRS plasmids (Sikorski and Hieter, 1989; Christianson et al., 1992). In the case of the \( ADE2 \) plasmids, pRS401, pRS411 and pRS421, the fragment inserted represents a minimal complementing fragment as previously determined by Stotz and Linder (1990). For the \( MET15 \) plasmids, pRS401, pRS411 and pRS421, a minimal length complementing fragment as defined by a complementation analysis (Figure 7). The \( NarI–Scal \) fragment was the smallest strongly-complementing fragment and was cloned into the pRS plasmid backbones. The \( ADE2 \) and the \( MET15 \) plasmids were demonstrated to fully complement the \( ade2\Delta \) and \( met15\Delta0 \) mutations as judged by colony growth on appropriate selective media. In the course of these experiments we noted that unlike all of the other auxotrophic designer deletion markers, the \( met15\Delta0 \) strain eventually (and at a low frequency) spawns slow-growing pseudo-\( Met^+ \) papillae when replica-plated as a thick patch on YPD medium to SD plates. Such growth is maximal at 22°C, drops dramatically at 25°C and decreases linearly from 30–37°C, where little growth is observed. These pseudo-\( Met^+ \) papillae are not true revertants because they form black colonies on lead plates, like the parental \( met15 \) mutant strain. Most importantly, these colonies do not grow when restreaked on methionine-free medium, so they are not true \( Met^+ \) cells. Our working hypothesis is that these colonies represent mutants that are more efficient at scavenging methionine or cysteine from adjacent cells. This phenomenon will be described in more detail elsewhere, but does not create much of a practical problem. Using standard LiOAc transformation procedures, these pseudo-\( Met^+ \) colonies did not cause any detectable background (i.e. no visible colonies on a control plate to which no pRS4X1 plasmid DNA had been added). In fact no colonies appeared on the no DNA plate even after 10 days of growth. Finally, we constructed the integrating plasmid pRS400, which contains the \( kanMX4 \) gene (Wach et al., 1994). This is useful for use as a template for PCR-mediated gene disruption. Available information on the
sequences and availability of the complete series of pRS400 series vectors is summarized in Table 5.

Universal primers for PCR-mediated gene disruption using pRS plasmid templates

A pair of primer segments was designed which can be universally used to amplify any selectable marker from the pRS set of plasmids (Figure 8). Addition of 40 nts of sequence specific to the gene of interest at the 5' ends of the primers results in a single set of 60 nt primers which can be used to delete the gene of interest with either URA3, TRP1, HIS3, LEU2, LYS2, ADE2, MET15 or kanMX4. We have used this strategy to disrupt a number of genes in our laboratories.
Table 5. The pRS400 series of vectors.

<table>
<thead>
<tr>
<th>Plasmid and type(^a)</th>
<th>Selectable marker</th>
<th>Accession numbers(^b)</th>
<th>Available from(^c)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS400 (I)</td>
<td>\textit{kanMX4}</td>
<td>U93713</td>
<td>Pending</td>
<td>Wach \textit{et al.} (1994), this work</td>
</tr>
<tr>
<td>pRS401 (I)</td>
<td>\textit{MET15}</td>
<td>U93714</td>
<td>ATCC 87473</td>
<td>This work</td>
</tr>
<tr>
<td>pRS411 (C)</td>
<td>\textit{MET15}</td>
<td>U93715</td>
<td>ATCC 87474</td>
<td>This work</td>
</tr>
<tr>
<td>pRS421 (2)</td>
<td>\textit{MET15}</td>
<td>U93716</td>
<td>ATCC 87475</td>
<td>This work</td>
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<td>\textit{ADE2}</td>
<td>U93717</td>
<td>ATCC 87477</td>
<td>This work</td>
</tr>
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<td>U93718</td>
<td>ATCC 87478</td>
<td>This work</td>
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<td>U93719</td>
<td>ATCC 87479</td>
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<td>U03454</td>
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<td>pRS426 (2)</td>
<td>\textit{URA3}</td>
<td>U03451</td>
<td>ATCC 77107</td>
<td>Christianson \textit{et al.} (1992)</td>
</tr>
<tr>
<td>(pRS317)(^d) (C)</td>
<td>\textit{LYS2}</td>
<td></td>
<td>ATCC 77157</td>
<td>Sikorski and Boeke (1990)</td>
</tr>
</tbody>
</table>

\(^a\) Plasmid type is in parentheses: I, integrating plasmid; C, centromeric \textit{(CEN/ARS)} plasmid; 2 \mu origin-containing plasmid.
\(^b\) DNA sequence accession number. DNA sequences are available from VectorBase (URL: http://biology.queensu.ca/~miseners/vector.html) or GenBank.
\(^c\) ATCC, American Type Culture Collection, Rockville, MD; ST, Stratagene, La Jolla, CA. Sets of pRS vectors are available in kit form from ATCC (pRS401, 411, 421, 87476; pRS402, 412, 422, 87480; pRS423, 424, 425, 426, 77108).
\(^d\) No pRS400 series plasmids exist for \textit{LYS2} at this time.

Other applications for the designer deletion strains and new plasmids

Although we have focused on the application of PCR-mediated gene disruption in this paper, we envision that the designer deletion alleles will be the alleles of choice for a very wide range of applications. They are ideal as transformation markers because they cannot revert (with the special exception of \textit{met15} noted above). The ability to knock out genes of interest by the PCR approach is enhanced by expanding the set of available markers in pRS vectors. This is especially useful in the study of multigene families (Brachmann \textit{et al.}, 1995) many of which have been revealed by the completion of the \textit{S. cerevisiae} genome sequence. By synthesizing a single pair of primers for each target gene of interest, the investigator has the ability to knock out a member of the gene family with any of eight currently available markers in the pRS vectors (Table 5). If a heavily marked strain such as BY418 is used as the recipient, up to eight different genes can be knocked out before running out of selectable markers. In addition, the alleles that have no overlap (or minimal overlap only at one end of the marker) will be ideal for studies of DNA recombination in which lack of homology between e.g. a plasmid and the chromosome is critical. It should be pointed out that an alternative and elegant strategy to solve the same problem was developed by Wach \textit{et al.} (1994), who constructed the \textit{kanMX4} marker, which has no homology with the yeast genome and confers dominant resistance to G418, and hence can be used with strains of any background. Although \textit{kanMX4} does work as a selectable marker, G418 is an expensive reagent to use routinely, and expression of the drug resistance requires an outgrowth period prior to plating on G418 and so is somewhat less convenient. Both strategies further enrich the already extensive molecular genetic toolkit available for \textit{S. cerevisiae}.  

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Figure 6. New pRS400 series \textit{S. cerevisiae–E. coli} plasmid vectors. Restriction maps of the pRS vectors. Unique restriction sites are shown in bold letters. For emphasis, sites in the polylinker that are no longer unique have been underlined. Maps of the pRS40X set may be converted to either the pRS41X set by the insertion of the \textit{CEN/ARS} cassette or to the pRS42X set by insertion of the 2\,\mu cassette (in either case, the \\textit{Aat}II site is destroyed). The numbering system is the same for all three sets. The direction of \textit{T} or \textit{T} polymerase transcription is as labeled. Numbers of parentheses refer to base pairs. Maps are drawn to scale.

Figure 7. Definition of the minimum complementing region of \textit{MET15}. A schematic diagram of the \textit{MET15} genomic region indicating relevant restriction sites. Lines below the restriction site diagram indicate the extent of the fragment tested for complementation of a \textit{met}\textsubscript{15} strain, and the column at the right indicates a qualitative analysis of the ability of each fragment to complement the \textit{met}\textsubscript{15} allele for growth. In all cases, the fragments were cloned into the polylinker of pRS413 and yeast (strain 31-10C; Cost and Boeke, 1996) transformants were selected on SD+Ura medium, and complementation analyses were performed by comparing colony sizes on selective medium between a wild-type \textit{MET15}\textsuperscript{+} strain and the transformed strain.
Figure 8. A universal primer set for producing PCR-mediated gene disruption cassettes. The double-stranded sequence shown represents about 50 bp surrounding a unique NdeI site in the parental plasmid backbone of all pRS400 series vectors [the parental backbone is a pBLUESCRIPT/pBLUESCRIPE hybrid made by ligating a PvuI fragment of pBLUESCRIPT II-SK (containing the polylinker region) to a PvuI fragment of pBLUESCRIPE (containing unique NdeI and AarII sites)]. Minimal DNA segments that encode each of the selectable markers (arrows indicate transcriptional orientation) shown above were blunt-end ligated into the blunted NdeI site for each pRS400 series vector. A single pair of oligos can therefore be used to amplify by PCR each of the selectable marker genes. Sequences of oligos used for amplification of the selectable marker genes are as indicated. Both oligos are drawn 5’ to 3’. As indicated, 40 nts of sequence from either the upstream or downstream flanking region of the gene to be deleted, in this case YFG, is added to the 5’ end of each oligo. PCR-amplification of the auxotrophic marker allele is performed using any pRS integrating vector as a template (this is possible because the oligos are specific to the sequence just upstream and downstream of the NdeI vector site into which all markers were introduced). The resulting double-stranded PCR product is then transformed into yeast, replacing the genomic YFG allele by a double cross-over event. The resulting disruptions contain any of eight selectable markers transcribed in the orientations indicated.
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