# Saccharomyces cerevisiae single-copy plasmids for auxotrophy compensation, multiple marker selection, and for designing metabolically cooperating communities

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METHOD ARTICLE

# *Saccharomyces cerevisiae* single-copy plasmids for auxotrophy compensation, multiple marker selection, and for designing metabolically cooperating communities [version 1; peer review: 2 approved]

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## Abstract

Auxotrophic markers are useful tools in cloning and genome editing, enable a large spectrum of genetic techniques, as well as facilitate the study of metabolite exchange interactions in microbial communities. If unused background auxotrophies are left uncomplemented however, yeast cells need to be grown in nutrient supplemented or rich growth media compositions, which precludes the analysis of biosynthetic metabolism, and which leads to a profound impact on physiology and gene expression. Here we present a series of 23 centromeric plasmids designed to restore prototrophy in typical *Saccharomyces cerevisiae* laboratory strains. The 23 single-copy plasmids complement for deficiencies in *HIS3, LEU2, URA3, MET17 or LYS2* genes and in their combinations, to match the auxotrophic background of the popular functional-genomic yeast libraries that are based on the S288c strain. The plasmids are further suitable for designing self-establishing metabolically cooperating (SeMeCo) communities, and possess a uniform multiple cloning site to exploit multiple parallel selection markers in protein expression experiments.

## Keywords

Saccharomyces cerevisiae , centromeric plasmid , auxotrophic markers , self-establishing metabolically cooperating (SeMeCo) communities , metabolism

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## Introduction

Auxotrophic markers are single gene perturbations of essential metabolic pathways, that are exploited in the efficient selection of strains, plasmids and genome editing. Further, they are used in a diverse spectrum of genetic technologies, as their selection is efficient, their use economic, and in contrast to antibiotic selection markers, they do not revert by mutation[1–3]. In budding yeast, auxotrophic marker alleles important for histidine, leucine, uracil, methionine, lysine, adenine and tryptophan metabolism have been crossed or cloned into the popular *S. cerevisiae* laboratory strains. Harbouring 5 auxotrophic marker mutations, *his3Δ1*, *leu2Δ0, ura3Δ0, lys2Δ0* or *met17Δ0*[4–9], strains derived from the S288c background served as the parents of the yeast gene-deletion collection[5], and subsequent genetic libraries that are based on this principle. These libraries include gene deletion mutants[5,10,11], genetically introduced GFP, GST, and TAP fusions[12–14], transposon insertion mutants[15], decreased abundance by mRNA perturbation (DAmP) mutants[16], Tet-promoter controlled expression[17] and the ts-alleles for essential genes[18,19]. Furthermore, systematic strain collections of other fungal species including *Schizosaccharomyces pombe*[20,21] and the pathogens *Candida glabrata*[22], *Candida albicans*[23] or *Neurospora crassa*[24], all involve use of auxotrophic markers as well. As a result, auxotrophic backgrounds are omnipresent in a large number of functional genomic experiments, and have been used in a countless number of small-scale experiments, resulting in their ubiquity across yeast molecular biology literature.

In order for a metabolic gene to function as an auxotrophic marker, it needs to be part of a metabolic pathway for which the cells possess an extracellular uptake and a sensing mechanism for the product of the interrupted pathway. Auxotrophic marker mutations are hence associated with metabolites that are readily taken up from the environment. This includes the metabolites exchanged between cells in communal growth, in particular amino acids[25]. The biosynthesis of amino acids accounts for up to half of the metabolic flux towards biomass, with amino acids making up to 2/3rds of the total mass of polar metabolites[26,27]. As a consequence, a shift from self-synthesis to uptake, as enforced by auxotrophy, is not without biological consequence. In fact, most of the genome-wide gene expression is sensitive to epistatic interaction within the *Saccharomyces* metabolic-genetic background[28]. The physiological effects arising from auxotrophy and complemented marker genes have been highlighted by several yeast labs for more than a decade[2,29–33]. Most importantly, to grow auxotrophic strains, amino acids and nucleotides need to be added to the growth medium. Nutrient supplementation affects not only the interrupted pathway itself, but the biosynthesis of other essential compounds, in particular the enzymatic cofactors, due to the metabolic network responding to perturbation at the systems levels, and hence, affecting multiple metabolic pathways in parallel[2,34,35]. Cell growth has consistently shown to be affected by nutrient supplementation, reflecting the variation in energy costs between biosynthesis and uptake/incorporation of the provided nutrients[2,34]. In batch cultures, supplements are also consumed at different rates[33]. As a consequence, nutrient availability changes during batch culture growth, rendering cells physiologically different between growth phases. In classic molecular biology, the use of a matched auxotrophic background as a wild-type control has been considered sufficient to account for the effects of auxotrophy[2]. Transcriptomic, proteomic and metabolomic analysis of complemented auxotrophs show however that this is not the case; the metabolic background deficiencies interact epistatically with the majority of the coding genome and in a context dependent manner. The biological explanation for this phenomenon is that metabolism is intrinsically intertwined with the gene expression machinery and is dependent on metabolic flux distribution. The same gene deletion introduced in a different auxotrophic background can hence cause an entirely different transcriptional response, so that a matched parent background is not able to compensate for these effects[28].

We here present a series of single copy plasmids derived from the pHLUM minichromosome, which can be used for restoring prototrophy as well as for testing the metabolic capacity of budding yeast, by compensating for the possible combinations of *his3*, *leu2*, ura*3*, *met17* (or *lys2*) deficiencies. For their use in *S. cerevisiae*, the plasmids contain a centromeric origin for single copy expression and express the marker genes under native *S. cerevisiae* promoter sequences[39]. To exploit multiple markers to reduce the plasmid segregation problem for protein expression experiments, we further introduced the uniform multiple cloning site of the pRS300 vector series. For cloning and manipulation in *E. coli*, the shuttle vectors contain a bacterial high-copy replication origin (pUC) and an ampicillin antibiotic resistance marker. Finally, the pHLUM series of the plasmid contains an N-terminal fragment (α-peptide) of the *E. coli* beta galactosidase (*lacZ*), for blue-white selection in appropriate cloning strains[40], and an F1 origin for use in phage libraries. These plasmids can be used for complementing unused auxotrophies in laboratory yeast stains, to express proteins exploiting multiple parallel selection markers, and to study metabolite exchange interactions in synthetic yeast communities.

## Materials and methods

### Strains, media and cultivation conditions

*Escherichia coli* strain *DH5α* was used as plasmid host and strains containing the recombinant plasmids were selected on LB medium with ampicillin (100 µg/ml) and grown at 37°C. Two commonly used *S. cerevisiae* strains in the S288c background, BY4741 (*MAT*a *his3Δ1 leu2Δ0 met17Δ0 ura3Δ0*) and BY4742 (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*)[4], were used to test for genetic complementation of auxotrophic requirements by the plasmids created. The strains were grown in YPD (2% Glucose, 20 g/l peptone (Bacto™), 10 g/l yeast extract (Bacto™)) or synthetic minimal (SM) medium (2% glucose, 6.8 g/l yeast nitrogen base), as indicated. To enable growth of auxotrophic strains, the SM medium was supplemented with 20 mg/l histidine, 60 mg/l leucine, 20 mg/l uracil, 20 mg/l methionine and/or 50 mg/l lysine as indicated.

### Plasmid construction

For site directed mutagenesis the Quikchange Lightning kit (Agilent) was used according to manufacturer guidelines, taking 50 ng of plasmid DNA as a template and 6.3 µM of each oligonucleotide (primers O09-O12, Table 1) to a total volume of 25 µl. The manufacturer's recommended cycling parameters, with a 2.5 min extension time, were followed.

**Table 1. List of oligonucleotides used to create pHLUM, pHLUM (version 2) and pHLUK.**

| Primer ID | Sequence (5'-to-3') | Application |
| --- | --- | --- |
| O01 | GACGGATCCTCGACTACGTCGTAAGGCCGT | amplification of *LEU2* from pRS425, *BamHI* restriction site in leader sequence |
| O02 | TCACTCGAGGGCGGCGCCATCGAGGAGAACTTCTAGTA | amplification of *LEU2* from pRS425, *AscI* and *XhoI* restriction sites in leader sequence |
| O03 | ATGGCGCGCCTGATGCGGTATTTTCTCCTT | amplification of *URA3* from p426GPD, *AscI* restriction site in leader sequence |
| O04 | GGCCTCGAGGCATGCGATTCGGTAATCTCCGAACA | amplification of *URA3* from p426GPD, *SphI* and *XhoI* restriction sites in leader sequence |
| O05 | ATCGCATGCGCCATCCTCATGAAAACTGT | amplification of *MET17* from pRS411, *SphI* restriction site in leader sequence |
| O06 | CATCTCGAGCTTGTGAGAGAAAGTAGGTT | amplification of *MET17* from pRS411, *XhoI* restriction site in leader sequence |
| O07 | TAGCGTCGACGCGTCGAGGAAAACTCTTCAATAG | amplification of *LYS2* from BY4741, *SalI* and *MluI* restriction sites in leader sequence |
| O08 | GCTAGCATGCACATATCATACGTAATGCTC | amplification of *LYS2* from BY4741, *SphI* restriction site in leader sequence |
| O09 | CTCTTGAACTCGAGGATCCTATGCGGTGTG | modification by site directed mutagenesis, new *XhoI* and *BamHI* restriction sites |
| O10 | GGTGTTGGCGGACGTCGGGGCTGGCTTAAC | modification by site directed mutagenesis, new *AatII* restriction site |
| O11 | CTAGAACTAGTGGGTCCCCCGGGCTG | modification by site directed mutagenesis, loss of *BamHI* restriction site |
| O12 | CGATACCGTCGACCTGGAGGGGGGGCC | modification by site directed mutagenesis, loss of *XhoI* restriction site |
| O13 | GATCTGTTTAAACTTAATTAACCTAGGA | modification by annealed primer cloning, loss of *BamHI* and addition of a *PmeI* restriction site |
| O14 | GATCTCCTAGGGTTAATTAAGTTTAAACA | modification by annealed primer cloning, loss of *BamHI* and addition of a *PmeI* restriction site |
| O15 | TCAGAGCAGATTGTACTGAGAGTGCACCATAATTCAAAATGACACCGATTATTTAAAGCTGCAGCATACGATAT | modification by homologous recombination, excision of HIS3 |
| O16 | ATATCGTATGCTGCAGCTTTAAAATAAATCGGTGTCATTTTGAATTATGGTGCACTCTCAGTACAATCTGCTCTGA | modification by homologous recombination, excision of HIS3 |

All other enzymes for molecular cloning were purchased from New England Biolabs (NEB) and used as instructed. Genomic DNA was extracted from yeast with repeated freeze-thawing of cells in a lysis buffer as per previous publication[36]. DNA from genomic and plasmid templates was amplified with Phusion High-Fidelity DNA Polymerase (Finnzymes) supplemented with the CES combinatorial enhancer solution to increase primer specificity as described previously[37].

Plasmids were isolated both from *E. coli* and *S. cerevisiae* with the QIAprep Spin Miniprep Kit (Qiagen). For the latter, a Qiagen protocol (Michael Jones, Chugai Institute for Molecular Medicine, Ibaraki, Japan, https://www.qiagen.com/gb/resources/resourcedetail?id=5b59b6b3-f11d-4215-b3f7-995a95875fc0&lang=en) was used. The protocol employs 425–600 µm acid-washed glass beads (Sigma) for mechanical lysis (30 sec, 6.5 M/s in a FastPrep®-24 Instrument (MP Biomedicals)). For homologous recombination to construct pLUK, two complementary primers were designed with 5' and 3' leader sequences homologous to plasmid 5' and 3' of the *HIS3* marker gene (primers O15-O16, Table 1). The oligonucleotides were annealed by heating to 95°C then gradual cooling to RT. The plasmid pHLUK was linearized by cutting inside the *HIS3* sequence with *Msc*I. The yeast strain BY4741 was transformed with 100 ng of cut vector and 500 ng of annealed primers and was selected on SM medium supplemented with histidine. Clones with successful homologous recombination events were identified by failure to grow on SM medium without histidine.

### Yeast transformation
Yeast strains were transformed with a high efficiency lithium acetate, PEG, salmon sperm protocol using 300 ng of plasmid per reaction[38].

## Results
### Generation of the plasmid backbone for pHLUM (version 2) series, to complement auxotrophies in BY4741 and isogenic strains
For the genetic complementation of the commonly used auxotrophic lesions in *HIS3*, *LEU2*, *URA3* and *MET17* we have previously constructed the pHLUM minichromome (Addgene ID 40276)[33]. The plasmid is based on pRS313[39], contains a centromer, and an autonomous replication sequence, and the *HIS3* marker as derived from the pRS313 backbone. The additional marker genes *LEU2*, *URA3* and *MET17* were cloned from other popular yeast plasmids (pRS425[39], p426GPD[41] and pRS411[4] and were placed between unique restriction sites, so that they can be individually excised[33], primers O01 - O06, Table 1). The three-gene insert is flanked by *Bam*HI and *Xho*I and unique restriction sites *Asc*I and *Sph*I were designed between *LEU2/URA3* and *URA3/MET17,* respectively, to allow for selective excision of the individual markers. However, in this original version of pHLUM, the *HIS3* marker cannot be removed in a straightforward manner.

In order to improve the usefulness of the minichromosome, we decided to redesign the plasmid backbone, replacing all 4 marker genes but leaving the multiple cloning site of pRS313 intact. With a site directed mutagenesis strategy, we added an *Aat*II restriction site 5' and *Xho*I and *Bam*HI sites 3' of *HIS3* (primers O09-O10, Table 1). In the same reaction we eliminated the *Bam*HI and *Xho*I recognition sites from the multiple cloning site by exchanging two

bases and preserving the sequence of the *lacZ* α-peptide (primers O11-O12, Table 1). With the new restriction sites available and the absence of *Xho*I and *Bam*HI in the multiple cloning site, the DNA fragment containing *LEU2*, *URA3* and *MET17* could be excised from pHLUM with *Xho*I and *Bam*HI and integrated 3' of the *HIS3* gene on the modified pRS313 vector. The resulting plasmid was named pHLUM (plasmid *HIS3 LEU2 URA3 MET15*) (version 2). It maintains 8 unique endonuclease recognition sites in the multiple cloning site and the capacity for colorimetric *lacZ* complementation assays (Figure 1A).

### Generation of the plasmid backbone for pHLUK series, to complement auxotrophies in BY4742 isogenic strains
In the typical *MAT*α derivatives of the *S. cerevisiae* gene deletion collection (i.e. BY4742), *LYS2* is deleted while the *MET17* marker is wild-type. We used pHLUM (version 2) as a template and exchanged the *MET17* marker with *LYS2* to create an analoguos vector series (pHLUK). The *LYS2* coding sequence contains recognition sites for both *Xho*I and *Bam*HI. We therefore removed the *Bam*HI site from pHLUM (version 2), and introduced at the same position a recognition sequence for *Pme*I. To this end we synthesised two complementary oligonucleotides (O13-O14, Table 1) and annealed them by heating to 95°C and gradual cooling to RT to yield a small double stranded DNA segment containing a *Pme*I site and cohesive ends to the *Bam*HI digested pHLUM (version 2). The digested vector was dephosphorylated with Antarctic phosphatase (NEB), the annealed primers phosphorylated with polynucleotide kinase (NEB) and then ligated by T4 DNA ligase abolishing the recognition site for *Bam*HI. Next, we amplified the *LYS2* gene from BY4741 genomic DNA (O07-O08, Table 1) including the promoter and terminator regions according to the yeast promoter atlas[42]. Primer O07 contained recognition sites for *Sal*I and *Mlu*I and primer O08 for *Sph*I (Table 1). The modified plasmid was digested with *Xho*I and *Sph*I and the *MET17* marker replaced with the *LYS2* PCR product digested with *Sal*I/*Sph*I. The cohesive ends of *Sal*I and *Xho*I DNA fragments are compatible and abolish their recognition sites upon ligation. The *Mlu*I site allows digestion of the vector between *LYS2* and *HIS3* and excision of either marker (Figure 1B).

### Generation of pHLUM and pHLUK derivatives possessing all marker combinations
The unique endonuclease recognition sites between each of the marker genes facilitated the creation of the 21 derivatives of pHLUM (version 2) and pHLUK containing between 1 and 3 marker genes, in all possible combinations. The marker genes were excised by digestion with appropriate endonucleases, the 3' and 5' overhangs were removed or filled in with T4 DNA polymerase and the plasmid ligated with T4 ligase (Table 2, Figure 2). The plasmid pLUK was generated using homologous recombination in yeast.

The completed plasmids were re-sequenced, which led to some corrections compared to the Genebank deposited version of pRS313 (GenBank: U03439.1) (Supplementary material). Successful genetic complementation of auxotrophic markers is illustrated upon transforming BY4741 and BY4742 strains, with the generated plasmids, and subsequent scoring of their growth on selective media for histidine, leucine, uracil and methionine or lysine, respectively. The plasmids restored all auxotrophies in BY4741 and BY4742 in the desired combination (Figure 3). Further, we tested the
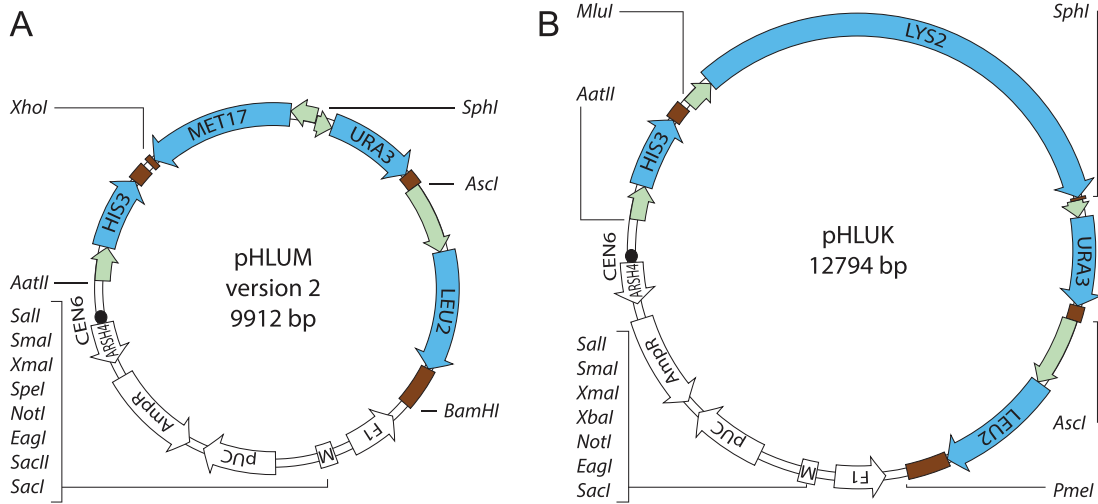
**Figure 1. pHLUM (version 2) and pHLUK.** Physical maps of pHLUM (version 2) and pHLUK minichromosomes, the centromeric parents for the generated *S. cerevisiae* vector series. pHLUM (version 2) expresses *HIS3, LEU2, URA3 and MET17* to complement auxotrophies in BY4741/*MAT*a strains of the knock-out collection, while pHLUK expresses *HIS3, LEU2, URA3 and LYS2*, for the BY4742/*MATα* series.

**Table 2. A plasmid series to complement auxotrophic markers *HIS3, LEU2, URA3, MET17* or *LYS2*, in 23 possible combinations.**

| Plasmid | Addgene ID | Marker genes | Parental plasmid | Cloning strategy |
|---|---|---|---|---|
| pHLUM (version 2) | 64166 | *HIS3 LEU2 URA3 MET17* | pRS313 and pHLUM (ID 40276) | SD, RE |
| pHLU | 64167 | *HIS3 LEU2 URA3* | pHLUM (version 2) | RE, BE |
| pHLM | 64168 | *HIS3 LEU2 MET17* | pHLUM (version 2) | RE, BE |
| pLUM | 64169 | *LEU2 URA3 MET17* | pHLUM (version 2) | RE, BE |
| pHUM | 64170 | *HIS3 URA3 MET17* | pHLUM (version 2) | RE, BE |
| pHL | 64171 | *HIS3 LEU2* | pHLUM (version 2) | RE, BE |
| pHU | 64172 | *HIS3 URA3* | pHUM | RE, BE |
| pHM | 64173 | *HIS3 MET17* | pHLUM (version 2) | RE, BE |
| pLU | 64174 | *LEU2 URA3* | pHLUM (version 2) | RE, BE |
| pLM | 64175 | *LEU2 MET17* | pLUM | RE, BE |
| pUM | 64176 | *URA3 MET17* | pHUM | RE, BE |
| pL | 64177 | *LEU2* | pHLUM (version 2) | RE, BE |
| pH | 64178 | *HIS3* | pHLUM (version 2) | RE, BE |
| pM | 64179 | *MET17* | pLUM | RE, BE |
| pU | 64180 | *URA3* | pLU | RE, BE |
| pHLUK | 64181 | *HIS3 LEU2 LYS2 URA3* | pHLUM (version 2) | AP, RE, BE |
| pHLK | 64182 | *HIS3 LEU2 LYS2* | pHLUK | RE, BE |
| pHUK | 64183 | *HIS3 URA3 LYS2* | pHLUK | RE, BE |
| pLUK | 64184 | *LEU2 URA3 LYS2* | pHLUK | HR |
| pUK | 64185 | *URA3 LYS2* | pHUK | RE, BE |
| pLK | 64186 | *LEU2 LYS2* | pHLK | RE, BE |
| pHK | 64187 | *HIS3 LYS2* | pHLUK | RE, BE |
| pK | 64188 | *LYS2* | pHK | RE, BE |

SD, site directed mutagenesis; RE, restriction endonuclease; BE, blunt end ligation; HR, homologous recombination; AP, annealed primer cloning
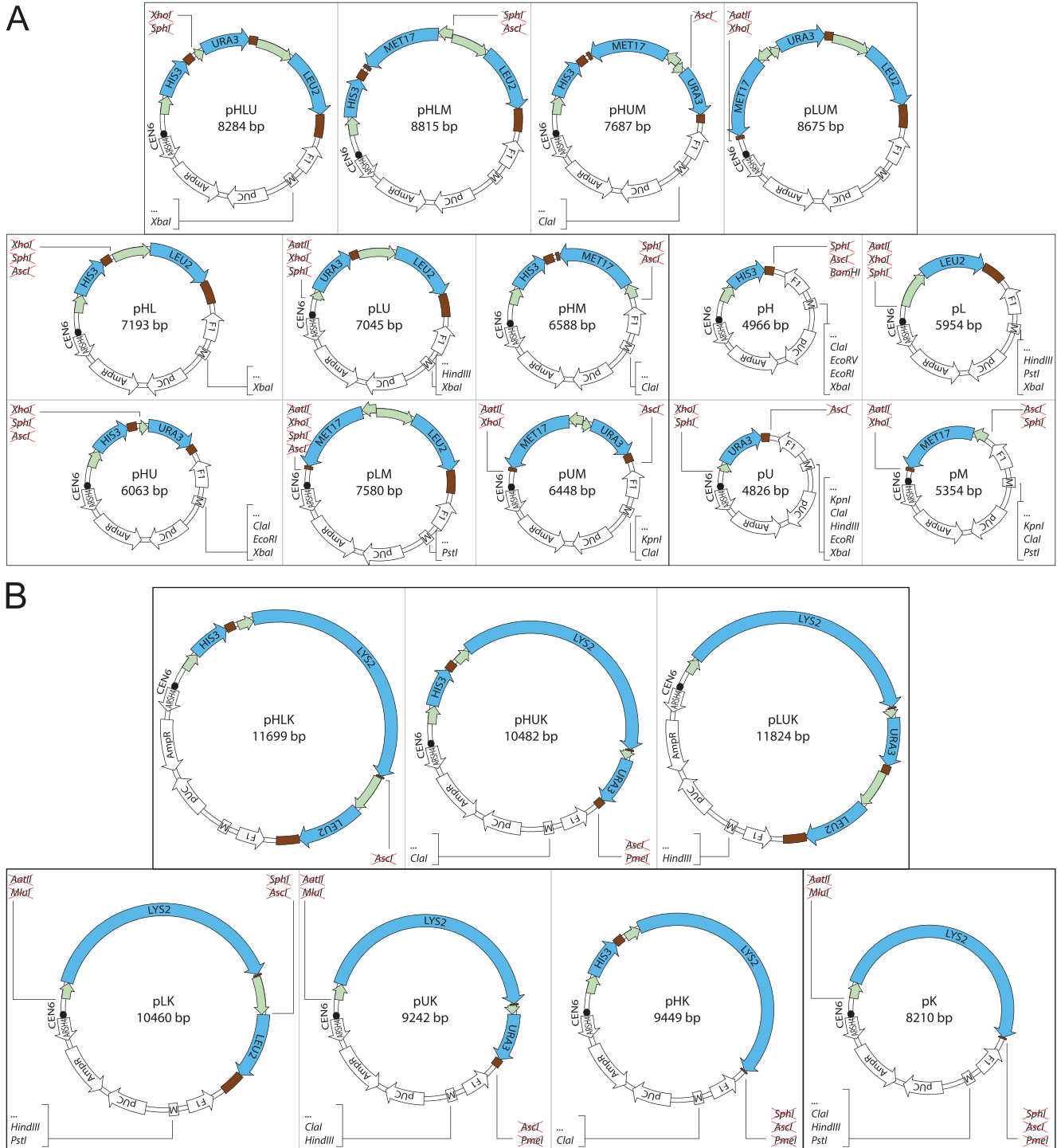
**Figure 2. A plasmid series to restore prototrophy in derivatives of BY4741 and BY4742. (A)** The plasmids are generated from pHLUM (version 2) containing *HIS3, LEU2, URA3* and *MET17*, and (**B**) pHLUK containing *HIS3, LEU2, URA3* and *LYS2* expressed under control of *S. cerevisiae* promoter and terminator sequences. Unique restriction sites between the marker genes and in the multiple cloning site (M) are indicated in the parent pHLUM (version 2) and pHLUK (Figure 1). Loss or acquisition of unique restriction sites is highlighted in the individual vector maps.
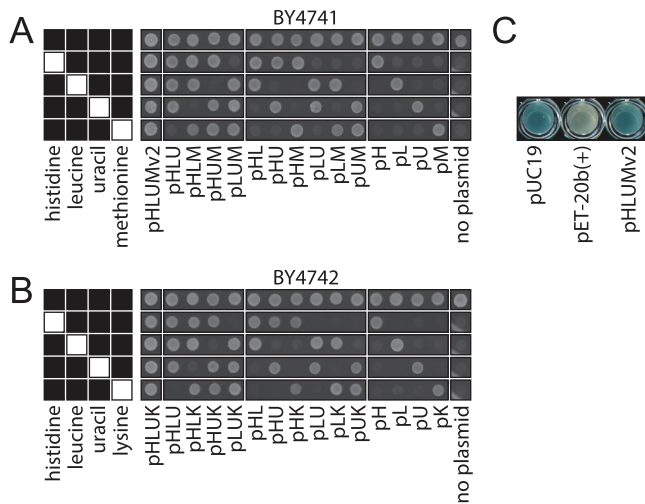
**Figure 3. Transformation of BY4741 and BY4742 with the 23 plasmids restores growth on selective media.** (**A**) *S. cerevisiae* haploid strains, BY4741 (*MAT*a *his3Δ1 leu2Δ0 met17Δ0 ura3Δ0*) and (**B**) BY4742 (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*)[4] were transformed with each of the 23 centromeric plasmids from the pHLUM (version 2) and pHLUK series and spotted onto synthetic minimal medium containing four or all five supplements of 20 mg/l histidine, 60 mg/l leucine, 20 mg/l uracil, 20 mg/l methionine or 50 mg/l lysine, as indicated in black in left hand key. (**C**) Use of the plasmid series for colorimetric *lacZ* assays: DH5α transformed with the *lacZ* containing plasmid pUC19, the *lacZ* missing plasmid pET-20b(+) and pHLUM (version 2) (pHLUMv2), parent of 23 plasmid series. A shift from white to blue on X-Gal containing LB medium due to the presence of a partial *lacZ* sequence.

functionality of the *lacZ* α-peptide sequence retained in the pHLUM (version 2) series for blue-white selection, by transforming them in *DH5*α (Figure 3C). On X-Gal containing medium, a blue colour shift was observed.

## Discussion

Due to the physiological impact of auxotrophy one would in an ideal world conduct all yeast experiments in prototrophic backgrounds and, if the objective of the experiment is to study a physiological process, use cells grown in minimal nutrient medium. However, most existing *Saccharomyces* lab strain resources are auxotrophic, and a majority of genetic techniques depend on the ability to select with genetic markers. The switch to antibiotic resistance markers is not a viable alternative to auxotrophies in many cases, as antibiotics can be expensive, are prone to persistence of sensitive cells, and by interfering with translation or transcription, have strong biological effects on their own[43,44]. We have noticed in our previous work, that a useful workaround, or compromise, for many applications is to complement the unused auxotrophic marker mutation with a multi-gene containing, single copy, centromeric single copy plasmid (minichromosome) that compensates for metabolic deficiencies present in the cell[28,33]. By nature, introducing an episome adds a new constraint due to its segregation. However, we found that the four metabolic genes on the pHLUM minichromosome provide selective advantage also under nutrient-rich growth

conditions, so that cells retain the vector even in the absence of selection pressure[33]. Further, we tested for copy number effects, and found that the expression of *HIS3, LEU2, MET15* and *URA3* from the minichromosome fully suffices the biosynthetic needs[33]. A situation in which all cells are provided with a high concentration of nutrients, as would occur with media supplementation, may also be less native to cells community where, usually, a certain fraction of cells are dependent on metabolite exchange[25,45]. For the typical experiment, the constraints arising from segregation of a single-copy minichromosome that restores auxotrophy, are hence much smaller compared to the problems caused by the use of nutrient supplemented media and auxotrophic strain backgrounds.

To support the work with prototrophic yeasts, we present here 23 minichromosomal vectors for restoring prototrophy in popular laboratory strains of budding yeast. These plasmids compensate for histidine (*HIS3*), leucine (*LEU2*), uracil (*URA3*), methionine (*MET17*) and lysine (*LYS2*) deficiencies and combinations thereof, which have been introduced into many yeast strains derived from the S288c background. Furthermore, the multiple cloning array is compatible with the widely used pRS300 plasmid series and provides unique restriction sites to facilitate cloning of genes of interest. The different marker genes of these vectors also enable expression analysis in various genetic backgrounds. The main intended application of these plasmids is to restore auxotrophy in laboratory strains and to be able to conduct experiments in minimal nutrient supplemented medium. In this way, the effect of amino acid and nucleotide biosynthetic metabolism, which is responsible for a major fraction of the metabolic flux of a cell, as well as has a profound impact on gene expression and physiology[28], can be studied.

Another application for these plasmids is to study metabolic cooperation in self establishing yeast communities (SeMeCo). It has been known for a long time that a subpopulation of plasmid free cells can co-grow alongside plasmid containing cells, despite using nutrient selection[2,46–48]. In our lab we have exploited this property to study metabolite exchange interactions between cells, and developed a system of self-establishing metabolically cooperating communities (SeMeCo) in which a series of auxotrophs cooperate to enable the growth of a yeast community[25,45]. This system exploits plasmid segregation, starting from an initially self-supporting cell, that grows progressively into an increasingly heterogeneous population, which is able to proliferate on the basis of nutrient exchange occuring between yeast cells. The progressive self-establishment overcomes a failure that is typically observed when yeast auxotrophs are forced to establish a bilateral cooperation. Other than through self-establishment, this lethality is overcome by yeast cells being genetically modified to artificially overproduce metabolites needing to be exchanged. The synthetic communities generated in this way have been intensively studied and serve as a model for ecological metabolite exchange interactions[49–52]. The new vector series, having multiple auxotrophic markers on single centromeric plasmids, can support the design of such communities, as it removes the likelihood of recombination occurring when multiple plasmids are used in parallel, to obtain the desired auxotrophic background.

Finally, the uniform multiple cloning site (MCS) in the plasmid series allows for the inclusion of marker proteins, such as GFP or beta-galactosidase, to track individual cell types in SeMeCo communities, that reveal phenotypic heterogeneity at the single cell level[45]. This MCS further allows the use of these plasmids for the recombinant expression of proteins. Here, one can profit from multiple auxotrophic markers on one plasmid to improve selection and reduce plasmid segregation rate, so that (as long as no disadvantageous protein is expressed) the plasmids can be maintained in rich medium in the absence of selection pressure[33]. This strategy of using multiple markers in parallel can further be exploited to increase selection pressure, to counteract the well-known issue of clonal selection phenotypes, emerging when overexpressing recombinant proteins.

In summary, to test both the effects of prototrophy as well as the metabolic capacity of budding yeast, to design self-establishing metabolically cooperating communities, and to profit from multiple selection markers when expressing proteins, we present a series of centromeric plasmids that can compensate for histidine (*HIS3*), leucine (*LEU2*), uracil (*URA3*), methionine (*MET17*) or lysine (*LYS2*) deficiencies in 23 possible combinations. These vectors are accessible individually (Table 2) or as a Kit from Addgene (www. addgene.org/kits/prototrophy/). We hope they benefit the community when analysing the importance of biosynthetic metabolism for gene function, gene expression, physiology and metabolite exchange.

## Data availability
The full sequences of the 23 plasmids are deposited in Addgene under the ID numbers listed in Table 1.

## Author contributions
MM designed the plasmids, MM, KC, OM, and FE constructed the plasmids, MM, KC, tested the plasmids, MR designed the study, MM, KC and MR wrote the paper.

## Competing interests
No competing interests were disclosed.

## Supplementary material

**Plasmid sequences used in Figure 1 and Figure 2.** Plasmid sequences for the 23 vectors have been assembled from the pHLUM and pRS300 vector sequences, respectively, and verified and corrected upon resequencing.

Click here to access the data.

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# Open Peer Review

## Current Peer Review Status: ✔ ✔

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**Version 1**

Reviewer Report 24 October 2016

✔ **Amy A. Caudy**

Donnelly Centre and Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada

The Ralser group has thoughtfully designed and created a comprehensive set of plasmids suitable for the complementation of various combinations of the widely used leucine (*leu2*), uracil (*ura3*), methionine (*met17*, formerly known as *met15*), histidine (*his3*), and lysine (*lys2*) markers. Auxotrophy can have significant effects on a variety of phenotypes, as shown by this group and others (and as well-described in the introduction). This new collection of plasmids provides a convenient source of auxotrophic markers supplied from their endogenous promoters on a CEN/ARS plasmid, which is near single-copy in most situations.

Not content to make a simple derivative of the pHLUM plasmid they previously created in order to build a prototrophic version of the yeast deletion collection, the group went to significant efforts to preserve several sites including BamHI and XhoI within the blue/white compatible multiple cloning site of their base vector. These plasmids have been fully sequenced in collaboration with Addgene, revealing a handful of mutations not present in the original (theoretical) sequence of the parent plasmid deposited in Genbank more than a decade ago.

One minor point could be mentioned for clarity -

The introduction mentions supplementation of "nucleotides" but it is important to note that yeast cells do not take up (phosphorylated) nucleotides directly. Instead, these are dephosphorylated by extracellular phosphatases to nucleosides and then imported. There are also transporters that can efficiently take in nucleobases such as uridine.

*Competing Interests:* No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 10 October 2016

✔

**Campbell Gourlay**
Kent University, UK

The paper nicely describes the generation and testing of a useful plasmid set that allows complementation of commonly used auxotrophic markers in the budding yeast system. The paper provides clear and adequate descriptions of their construction and therefore represents a solid piece of work that is ready for indexing as it is currently presented.

*Competing Interests:* No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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