GENETICS

Acetamidase gene as a new plasmid marker for CRISPR modification of industrial and laboratory *Saccharomyces* yeasts

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Abstract

Genetic manipulation of laboratory yeast strains relies on the use of auxotrophic markers. However, such markers are usually missing and undesirable in industrial yeasts. The standard option is then to use antibiotic resistance markers such as KanMX. However, the required antibiotic concentration can vary significantly depending on the growth medium and yeast strain, often resulting in a high proportion of false-positive colonies. An alternative selection procedure could be based on the ability of yeast cells to utilize an uncommon source of nitrogen. Many yeasts, including *Saccharomyces*, are unable to utilize acetamide. This ability can be conferred by a single acetamidase gene. The CRISPR/Cas9 technology has an advantage over previous methods of yeast modification in that it allows genomic modifications to be introduced without leaving a selectable marker in the genome. Driven by these two motives, we constructed a CRISPR/Cas9 plasmid with an acetamidase gene from the fungus *Aspergillus nidulans*, which allows the selection of yeast transformants on medium with acetamide as sole nitrogen source, and tested this plasmid with a triploid wine strain and a haploid laboratory strain.

Keywords: wine yeast, selective marker, acetamidase gene, *CAR1* gene, CRISPR/Cas9, acetamide, arginine, nitrogen source.

Introduction

Genetic modification of yeast requires the use of selective markers to distinguish the cells that have received the genes of interest. In laboratory strains of *Saccharomyces cerevisiae*, selection is based on the use of auxotrophic markers, usually genes involved in amino acid or nucleotide biosynthesis, such as *URA3*, *LEU2* or *HIS3*. The pre-existing deficiency in such genes is compensated for by the incoming DNA, which carries both the information of interest and the corresponding wild-type gene for selection. Less commonly, selective markers allow cells to grow on specific carbon, nitrogen or phosphorus sources (for review, see (Siewers, 2022)).

In contrast to laboratory strains, industrial strains do not usually carry auxotrophic mutations, as these reduce their growth characteristics. Furthermore, it is usually required that no such mutation remains after all desired changes have been introduced. The remaining option is to use genes that provide the cells with novel abilities that are lacking in wild-type yeast, such as G418 antibiotic resistance, which is provided by the KanMX cassette. However, a common problem with this and other antibiotic resistance markers is that the sensitivity to antibiotics can vary significantly depending on both the yeast strain and the medium used, so transformations with KanMX often produce a high proportion of false-positive colonies as observed by (Fairlie, Russell, Zhang, and Breit, 1999) and in our experience. An alternative marker that does not have this drawback could be the acetamidase gene. Wild type *S. cerevisiae* cannot catabolize acetamide and

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therefore cannot grow on acetamide as a sole nitrogen source. Such an ability can be provided by a single extra gene, the acetamidase gene, which is found, for example, in the filamentous fungus *A. nidulans*.

As mentioned above, once all the desired modifications have been made, it is usually required that no foreign genes remain apart from the genes of interest. This makes the rapidly developing CRISPR/Cas technology the method of choice, as the selective marker is not associated with the introduced DNA of interest and can be easily lost from the modified cells. Another advantage of the CRISPR/Cas method relates specifically to polyploid yeasts, which make up the majority of industrial yeast strains. A classical cassette for chromosome integration contains the DNA of interest and a marker gene and can therefore only select for a single integration event. Targeting two, three or more gene copies is therefore very problematic. In contrast, CRISPR/Cas makes double-stranded breaks at all target sites, and a cell will not survive unless these breaks are repaired. Repair can occur either by homologous recombination with a DNA that can encode the desired change, or by non-homologous end joining. Fortunately, the latter is usually less common in *S. cerevisiae*. Though the use of the acetamidase as a marker for *S. cerevisiae* was proposed earlier (Hamilton et al., 2020; Solis-Escalante et al., 2013), it is still used very rarely and was never used in conjunction with the CRISPR/Cas technology.

We therefore created and tested a new CRISPR/Cas9 vector that enables *Saccharomyces* yeasts to assimilate acetamide as a nitrogen source. The efficacy of this vector, which harbors the acetamidase gene from *A. nidulans*, was demonstrated for industrial and laboratory strains of *S. cerevisiae*.

Materials and methods

Yeast strains, cultivation conditions and transformation

This study used the industrial triploid *S. cerevisiae* strain I-328 deposited in the Collection of Winemaking Microorganisms "Magarach" (Kishkovskaia et al., 2017; Mardanov et al., 2018) and the laboratory haploid strain 74-D694 (Chernoff et al., 1995). Yeast strains were grown at 30 °C in rich (YPD, 1 % yeast extract, 2 % peptone, 2 % glucose) or synthetic (SC, 0.17 % yeast nitrogen base without ammonium sulphate, 2 % glucose, 0,1 % K_2SO_4 and indicated amounts of acetamide or arginine as nitrogen source) media with 2.5 % agar. For the selection of CRISPR transformants of I-328, the SC+acmd medium containing 0.5 % acetamide was used. For the strain 74-D694, the SC+acmd medium also contained standard concentrations of uracil (20 mg/l), leucine (60 mg/l), histidine (20 mg/l) and tryptophan (20 mg/l), but a reduced concentration of adenine (6 mg/l, or 30 % of the standard value) for better manifestation of red colony color. To distinguish *Δcar1* strains, SC+arg medium containing 0.3 % arginine as the only nitrogen source was used.

Plasmids and nucleic acid manipulation

The plasmids and primers used in this study are listed in the Tables 1 and 2. To create a yeast CRISPR/Cas9 plasmid with an acetamidase marker gene, we started with the yeast multicopy pWS172 plasmid (Addgene #90519) encoding Cas9, guide RNA and the *HIS3* marker gene (Shaw, 2018). Notably, the guide RNA sequence is interrupted by the superfolder GFP gene, which is intended to be replaced by a specific targeting sequence of interest. This plasmid was cut at the SpeI and Acc65I sites to remove *HIS3* and insert the acetamidase gene. The amdS acetamidase gene from *A. nidulans*, but with the yeast *ADH1* promoter, was amplified by PCR from pKlac2, the integrative plasmid intended for expression in *Kluyveromyces lactis* (New England Biolabs, USA) (Colussi and Taron, 2005). One problem to solve was that the amdS gene contains an Esp3I (BsmBI) restriction site, which is used in pWS plasmids to insert a spacer that defines the Cas9 targeting specificity. This site in amdS was removed by changing the Esp3I recognition sequence from GAGACG to GAAACA without altering the encoded protein. To make this change, two halves of the amdS gene were PCR amplified from the pKLAC2 plasmid using the primers listed in Table 2. The PCR fragments overlapped by 20 bases and this overlap

Table 1. Plasmids used

Plasmid	Description	Source
pWS172	Yeast multicopy Cas9-sgRNA vector, *HIS3*	(Shaw, 2018), Addgene #90960
pWS-amdS	Yeast multicopy Cas9-sgRNA vector, amdS	This work
pWS82	sgRNA entry vector	(Shaw, 2018), Addgene #90516
pWS82-CAR1–3	sgRNA entry vector for *CAR1* gene	Urakov et al., 2023
pWS82-Sup35Cr1	sgRNA entry vector for *SUP35* gene	This work
pKlac2	Source of the amdS gene cassette	New England Biolabs

Table 2. Oligonucleotides used in this work

Primer	Sequence	Description
172Adh1-Df	CAGAGATGTTACGAACCACTAGTACAATATGGACTTCCTCTT	Junction of pWS172 and *ADH1* promoter
amdS172-Rf	GGAGGGAACATCGTTGGTACTATGGAGTCACCACATTTC	Junction of amdS and pWS172
amdSE3-Df	GGTCAGGAAACAGTGCACAGCGTTGTCGG	Removal of the Esp3I site inside amdS
amdSE3-Rf	CTGTGCACTGTTTCCTGACCCTCCATGCT	Removal of the Esp3I site inside amdS
Sup35-Cr1D	*GACT*CTGCCCACTAGCAACAATGT	Spacer for pWS82-Sup35Cr1
Sup35-Cr1R	*AAAC*ACATTGTTGCTAGTGGGCAG	Spacer for pWS82-Sup35Cr1
Car_1F	AACCGTGTAGGCAAAACTGGAC	*CAR1* deletion test
Car_4R	AGATGGCCGATTTGAGAGCCT	*CAR1* deletion test
Sup35-D1	CACTCGACCAAAGCTCCCA	*SUP35* deletion test
Sup35-seq1R	GGGTTCTTTGGCGATGTTAG	*SUP35* deletion test

Note: Complementary regions are underlined. Residues forming sticky ends for ligation into Esp3I sites are given in italics.

encoded the altered Esp3I site. The PCR fragments also overlapped by 20 nucleotides with the pWS172 plasmid cut at the SpeI and Acc65I sites. Such overlaps allow the DNA to be joined seamlessly using a ligase free technology. The described PCR fragments and the Acc65I-SpeI fragment of pWS172 were joined using the Quick-fusion cloning kit (Vazyme, China) and used to transform *E. coli*, resulting in the pWS-amdS plasmid (Fig. 1).

To verify genomic changes in yeast, DNA was isolated from yeast according to Looke, Kristjuhan, and Kristjuhan (2011) and amplified with primers to *CAR1* or *SUP35* (Table 2).

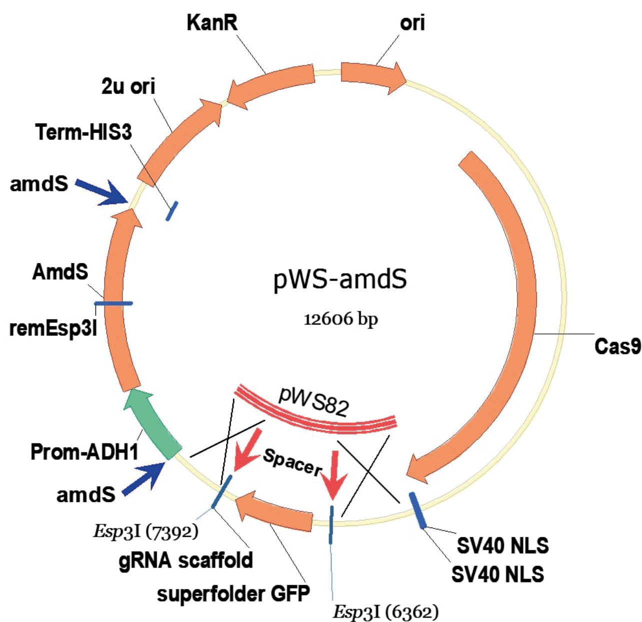

Fig. 1. The pWS-amdS plasmid. The blue arrows indicate the insertion sites of the *ADH1* promoter-amdS gene cassette. Red arrows: 20 nt spacer targeting sequences should be inserted here instead of sGFP through Esp3I sites, either by ligation or by homologous recombination with a similar fragment of the pWS82 plasmid loaded with a relevant spacer (Show18). RemEsp3I — Esp3I site removed.

Yeast genome modification

Alterations of yeast genome using pWS-amds plasmid were performed according to (Shaw, 2018). Briefly, the targeting sequence (spacer) has to be inserted by the Esp3I sites of this plasmid (Fig. 1). This can be accomplished by either directly ligating the spacer, or through homologous recombination in vivo of pWS-amdS with a homologous EcoRV fragment of pWS82 plasmid, where the appropriate spacer is inserted. For the latter scenario, transforming DNA included pWS-amdS plasmid cut with Esp3I and the EcoRV fragment of pWS82 with a spacer, as well as the DNA repair cassette encoding the required chromosomal alterations. Yeast cells were transformed with plasmids as described by Gietz and Woods (2002).

Results

Construction of the *Δcar1* polyploid yeast strains with the new pWS-amdS vector

To test the efficiency of the pWS-amdS plasmid with the industrial *S. cerevisiae* yeast, we chose to delete the *CAR1* gene in the triploid wine strain I-328 (Mardanov et al., 2018). This gene was chosen because the deletion of all its copies can be conveniently confirmed by the inability to assimilate arginine as the nitrogen source (Urakov et al., 2023). This deletion is also known to reduce the carcinogen ethyl carbamate in wine (Chin et al., 2021). The yeast was transformed as described in the Materials and methods. The transformation mix contained the EcoRV fragment of the pWS82-CAR1-3 plasmid and the Esp3I fragment of the pWS-amdS plasmid, as well as the DNA repair cassette. The Cas9 chromosomal cleavage was targeted to the nucleotide +5 of the *CAR1* open reading frame. The DNA repair cassette

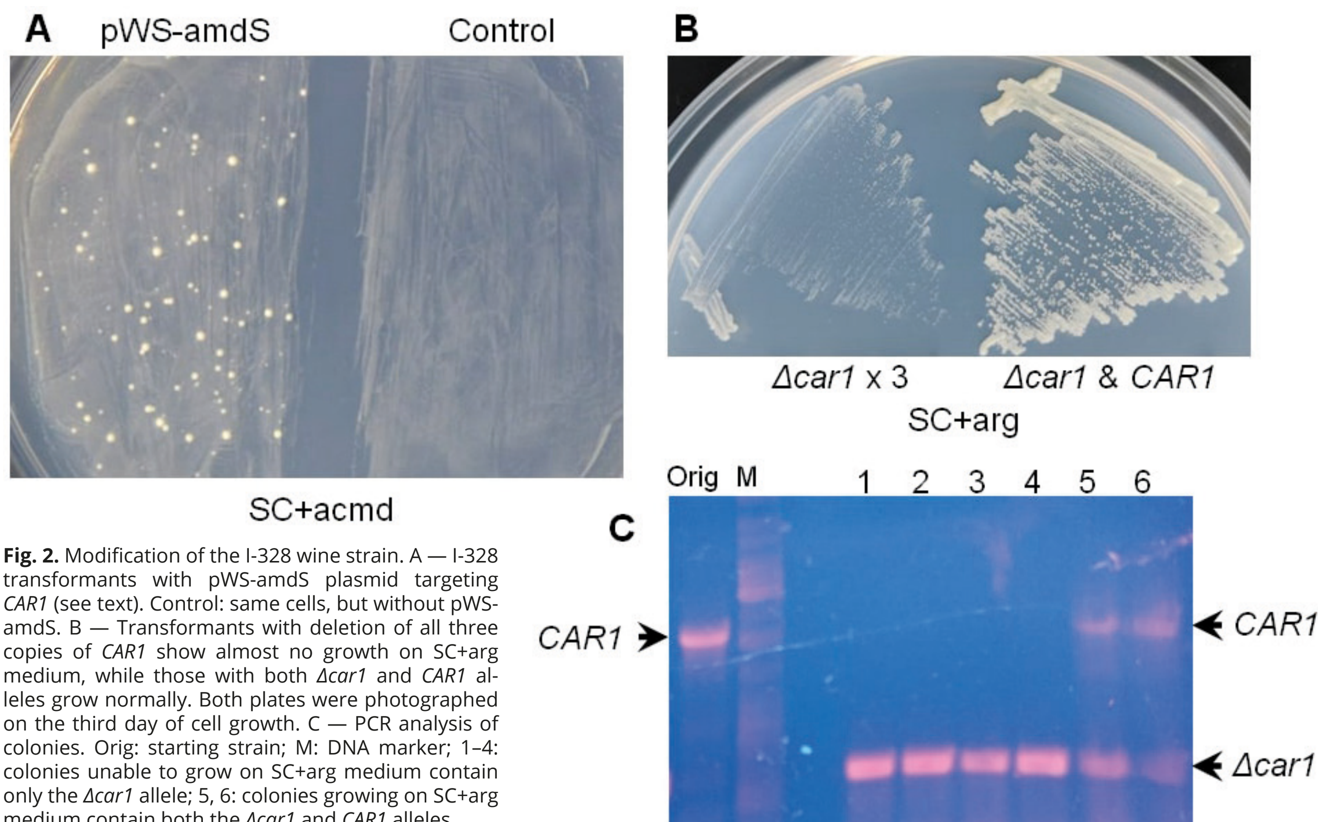

Fig. 2. Modification of the I-328 wine strain. A — I-328 transformants with pWS-amdS plasmid targeting *CAR1* (see text). Control: same cells, but without pWS-amdS. B — Transformants with deletion of all three copies of *CAR1* show almost no growth on SC+arg medium, while those with both *Δcar1* and *CAR1* alleles grow normally. Both plates were photographed on the third day of cell growth. C — PCR analysis of colonies. Orig: starting strain; M: DNA marker; 1–4: colonies unable to grow on SC+arg medium contain only the *Δcar1* allele; 5, 6: colonies growing on SC+arg medium contain both the *Δcar1* and *CAR1* alleles.

encoded a deletion of the *CAR1* coding region together with 521 nucleotides of the promotor and six nucleotides of the terminator regions.

Yeast transformants were selected on SC+acmd plates (Fig. 2). The pWS-amdS plasmid showed a high transformation efficiency comparable to that of the standard YEplac vectors (Gietz and Sugino, 1988). Fifty transformants were tested for their ability to grow on the SC+arg agar. Six clones, or 12 %, did not grow on this medium, indicating deletion of all *CAR1* copies. Complete and correct *CAR1* deletion in these clones was confirmed by PCR, while other clones tested contained both deleted and intact *CAR1* alleles (Fig. 2). In addition, the deletion of all *CAR1* copies was confirmed in one arginine auxotrophic transformant by whole genome sequencing. The sequence did not contain any additional changes other than the *CAR1* deletion and was identical to the previously deposited sequence (PEJR00000000) (Urakov et al., 2023).

Modification of a laboratory strain

While we expect the pWS-amdS plasmid to be most useful with industrial yeasts, it can also be used with laboratory strains. When working with laboratory yeasts, there are often situations where the available markers are already used up by some other DNA constructs and additional markers are needed. The acetamidase marker would be helpful in such cases, but it is surprisingly rarely used in laboratory practice. To test the performance of the pWS-amdS plasmid with haploid yeast strains, we used the 74-D694 strain (Chernoff et al., 1995). This strain contains a nonsense mutation *ade1–14* in the *ADE1* gene and is commonly used as an indicator of impaired translation termination, which occurs when the Sup35 (eRF3) translation termination factor is mutated or acquires the prion state. The 74-D694 strain forms red ade- colonies due to disruption of adenine biosynthesis pathway, resulting in the accumulation of a red-colored precursor (Nevzglyadova, Mikhailova, and Soidla, 2022). Sup35 mutations or aggregation into prion particles reduce the efficiency of translation termination and allow partial readthrough of the nonsense mutation in *ADE1*, resulting in white or pink colonies. For the testing, we used the 74-D694 strain with the Sup35 prion, usually referred to as [*PSI*+]. We used a so-called S1 “strong” variant of the prion, which is characterized by efficient nonsense codon readthrough and accordingly, white colony color (Dergalev et al., 2019). The Sup35 prion aggregation depends on the presence of inessential Sup35 N-terminal prion-forming domain (residues 2–123). Deletion of this domain eliminates the prion, which can be observed as reversion of the white colony color to red. To delete the Sup35 N domain, we directed Cas9 to the first ATG codon of the *SUP35* coding sequence. The DNA repair cassette contained the *SUP35* promoter and

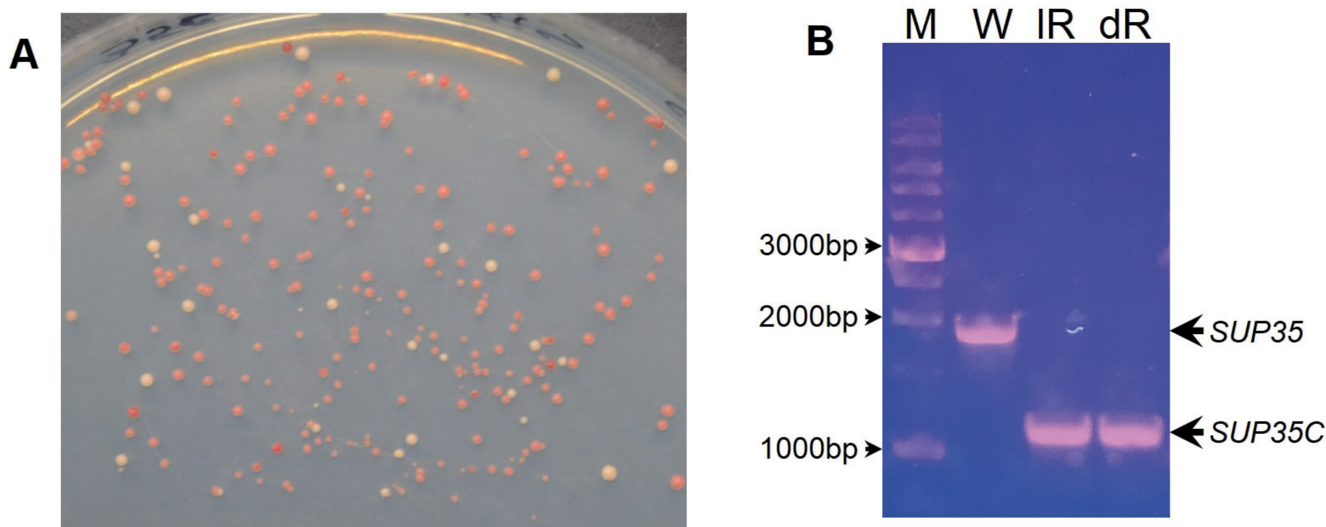

Fig. 3. Deletion of the *SUP35* prion domain causes loss of the Sup35 ([*PSI*+]) prion and reversion of the colony color from white to red. A — Colonies of transformants. Sup35 deletion was performed as described in the text. The plate was photographed on the fourth day after transformation. B — PCR analysis of colonies. PCR was performed with colonies of the following colors: W: white, lR: light red, dR: dark red. M: 1Kb DNA ladder (Sibenzyme).

the coding region for the Sup35 functional C-terminal domains, while the region encoding residues 1–253 was missing and the "target" sequence before the first codon was altered (Ter-Avanesyan et al., 1993).

While we suggested above to use acetamidase selection when other markers are used up, in our test we used the 74-D694 strain which has several auxotrophic markers, and thus we had to supplement the acetamide selection medium with leucine, histidine, tryptophan, uracil and adenine. This posed an additional challenge, because these amino acids and nucleotides could be used as alternative nitrogen sources. Nevertheless, we observed only negligible growth of non-transformed cells. This is probably because the standard concentrations of the compounds were not sufficient for cell growth.

Of the 237 transformants obtained, 204 (86 %) were light or dark red (Fig. 3). Several each of light and dark red and white transformants were tested by PCR. In the white transformants, *SUP35* was apparently unchanged, while the light and dark red transformants lacked the region corresponding to the N-terminal part of Sup35 to the same extent as the DNA repair cassette used (Fig. 3). A possible reason for the different shades of red colonies is that *SUP35* mutations often cause some kind of mitochondrial dysfunction, which manifests as darker brownish colonies.

Discussion

Our results show that the pWS-amdS plasmid can be used as an efficient tool for transformation of both industrial and laboratory *Saccharomyces* strains. While we see this plasmid primarily as an alternative to the KanMX-based plasmids such as pWS173 (Shaw, 2018), it could be particularly useful in projects involving the modification of multiple genes. Several strategies using CRISPR/Cas have been proposed that allow multiple genomic modifications to be made in a single transformation event (Jakočiūnas et al., 2015; Mans et al., 2015; Ryan et al., 2014; Zhang et al., 2019). They are based on the simultaneous introduction, in one way or another, of guide RNAs with different targeting specificities. However, it is doubtful whether these strategies can work with sufficiently high efficiency to justify their complexity. The efficiency of genomic modification at any given locus very rarely reaches 100 %. In our practice, the probability of correct modification of a locus varied from 30 to 90 %, depending on a target. Then, the probability of correct simultaneous modification of several loci could fall well below 10 %, especially when considering a polyploid industrial strain. A substantial amount of screening would be required, or, more likely, additional rounds of transformation with clones, in which some, but not all of the required changes occurred. The strategy developed by Shaw (2018) also promises to achieve multiple modifications by simultaneously including multiple target specificities in the transformation mix, represented by pWS82 fragments loaded with different spacers. Although we find his plasmid system very convenient, we doubt that more than one target at a time can be efficiently modified in this way, because after the first pWS82 fragment recombines with the "mother" CRISPR/Cas9 plasmid to make it circular and functional, there would be no further selection to create other target specificities by recombination in the same cell.

In our opinion, a more practical and simpler approach for modifying multiple targets would be to sequentially introduce different target sequences on two

CRISPR/Cas9 plasmids with different selective markers instead of complex schemes. In each round, correct transformants should be identified by PCR and then used for the next transformation using a CRISPR/Cas9 plasmid with a different marker. This avoids wasting time for the loss of the previous plasmid and thus one round can be completed in one week. The other marker, in addition to amdS, could be KanMX, or *CAR1* in the case of wine yeast, or any other auxotrophic or antibiotic resistance marker. Overall, the pWS-amdS plasmid described here may prove to be useful in many different ways.

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