amdSYM, a new dominant recyclable marker cassette for Saccharomyces cerevisiae

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Abstract

Despite the large collection of selectable marker genes available for Saccharomyces cerevisiae, marker availability can still present a hurdle when dozens of genetic manipulations are required. Recyclable markers, counterselectable cassettes that can be removed from the targeted genome after use, are therefore valuable assets in ambitious metabolic engineering programs. In the present work, the new recyclable dominant marker cassette amdSYM, formed by the Ashbya gossypii TEF2 promoter and terminator and a codon-optimized acetamidase gene (Aspergillus nidulans amdS), is presented. The amdSYM cassette confers S. cerevisiae the ability to use acetamide as sole nitrogen source. Direct repeats flanking the amdS gene allow for its efficient recombinative excision. As previously demonstrated in filamentous fungi, loss of the amdS marker cassette from S. cerevisiae can be rapidly selected for by growth in the presence of fluoroacetamide. The amdSYM cassette can be used in different genetic backgrounds and represents the first counterselectable dominant marker genetic cassette for use in S. cerevisiae. Furthermore, using astute cassette design, amdSYM excision can be performed without leaving a scar or heterologous sequences in the targeted genome. The present work therefore demonstrates that amdSYM is a useful addition to the genetic engineering toolbox for Saccharomyces laboratory, wild, and industrial strains.

Introduction

The past decade has been marked by the construction of complex cell factories resulting from dozens of genetic manipulations and leading to remarkable new capabilities. For instance, the industrial and model yeast Saccharomyces cerevisiae has been engineered to produce the anti-malaria drug precursor artemisinic acid (Ro et al., 2006), hydrocortisone (Szczepanska et al., 2003), and cineole (Suzuki et al., 2011), among others. The ever-increasing demand for cheap and sustainable production of complex molecules combined with its attractiveness as a host for pathway engineering will inevitably intensify the exploitation of S. cerevisiae as cell factory in the future (Hong & Nielsen, 2012). Also, in fundamental yeast research, extensive genetic manipulation is necessary, for instance to unravel complex transport systems (Wieczorke et al., 1999; Suzuki et al., 2011) and regulatory networks (Baryshnikova et al., 2010). A common and critical feature for all these genetic manipulations is the requirement of selectable markers that enable the selection of mutants carrying the desired genetic modifications. Despite the relatively large number of selection markers available for S. cerevisiae (Table 1), the construction of multiple successive genetic modifications remains a challenge as the number of genetic manipulations typically equals the number of selection markers introduced in the host. Selection markers can be classified in two main categories: auxotrophic markers, which restore growth of specific mutants, and dominant markers, which confer completely new functions to their host. Both types suffer from substantial drawbacks. The use of auxotrophic markers is restricted to auxotrophic strains, that is, strains carrying mutations in one gene leading to a strict requirement for a specific nutrient (Pronk, 2002). This constrain is augmented for industrial strains that are typically...
prototrophic and for which the aneuploidy or polyploidy makes the construction of auxotrophic strains a laborious task (Puig et al., 1998). The expression in a single strain of multiple dominant marker genes, under the control of strong promoters, may result in protein burden and other negative effects on host strain physiology (Gopal et al., 1996). Additionally, for industrial strains dedicated to food applications such as the production of nutraceuticals, the lack of heterologous DNA is highly desired.

While protein burden might be avoided by expressing marker genes from inducible promoters (Suzuki et al., 2011), many inducible promoters are notoriously leaky (Agha-Mohammadi et al., 2004) and therefore only partly address the problem. A good alternative resides in the use of recyclable markers. Marker recycling was first shown with \textit{URA3} (Alani et al., 1987). Loss of \textit{URA3}, encoding orotidine-5'-phosphate decarboxylase involved in pyrimidine biosynthesis, is lethal in uracil-free media. Since its discovery, \textit{URA3} has become a very popular auxotrophic selection marker. This popularity mainly originates from the ability of \textit{URA3} to be counter-selected in the presence of 5-fluoro-orotic acid (5-FOA), which is converted to a toxic compound (5-fluoro-UMP) by Ura3p. Indeed, when the \textit{URA3} marker is flanked by cassette sequences (or scars) are left (Alani et al., 1997), which is converted to a toxic compound (5-fluoro-UMP) by Ura3p. Indeed, when the \textit{URA3} marker is flanked by cassette sequences (or scars), they are left.

Table 1. Different selectable markers used in laboratory and industrial \textit{Saccharomyces cerevisiae} strains

| Marker gene | Mode of action | Recyclable/Method | References |
|-------------|----------------|-------------------|------------|
| \textit{URA3} | Repairs uracil deficiency | Yes/negative selection with 5-FOA | Alani et al. (1987), Langlerouault & Jacobs (1995) |
| \textit{KIUURA3} | Repairs uracil deficiency | Yes/negative selection with 5-FOA | Shuster et al. (1987) |
| \textit{CaURA3} | Repairs uracil deficiency | Yes/negative selection with 5-FOA | Losberger & Ernst (1989) |
| \textit{HIS3} | Repairs histidine deficiency | No/— | Wach et al. (1997) |
| \textit{HIS5} | Repairs histidine deficiency | No/— | Wach et al. (1997) |
| \textit{LEU2} | Repairs leucine deficiency | No/— | Brachmann et al. (1998) |
| \textit{KLEU2} | Repairs leucine deficiency | No/— | Zhang et al. (1992) |
| \textit{LYS2} | Repairs lysine deficiency | Yes/negative selection with alpha-amino adipate | Chattoo et al. (1979) |
| \textit{TRP1} | Repairs tryptophan deficiency | No/— | Brachmann et al. (1998) |
| \textit{ADE1} | Repairs adenine deficiency | No/— | Nakayashiki et al. (2001) |
| \textit{ADE2} | Repairs adenine deficiency | No/— | Brachmann et al. (1998) |
| \textit{MET15} | Repairs methionine deficiency | Yes/negative selection with methyl-mercury | Singh & Sherman (1974), Brachmann et al. (1998) |

Auxotrophic markers

| Marker gene | Mode of action | Recyclable/Method | References |
|-------------|----------------|-------------------|------------|
| \textit{KanMX} | Resistance to G418 | No/— | Wach et al. (1994) |
| \textit{ble} | Resistance to phleomycin | No/— | Gatignol et al. (1987) |
| \textit{Sh ble} | Resistance to Zeocin | No/— | Drocourt et al. (1990) |
| \textit{hp} | Resistance to hygromycin | No/— | Griz & Davies (1983) |
| \textit{Cat} | Resistance to chloramphenicol | No/— | Hadfield et al. (1986) |
| \textit{CUP1} | Resistance to Cu²⁺ | No/— | Henderson et al. (1985) |
| \textit{SFA1} | Resistance to formaldehyde | No/— | Van den Berg & Steensma (1997) |
| \textit{dehH1} | Resistance to fluoroacetate | No/— | Van den Berg & Steensma (1997) |
| \textit{PDR3-9} | Multi drug resistance | No/— | Lackova & Subik (1999) |
| \textit{AUR1-C} | Resistance to aureobasidin | No/— | Hashida-Okaodo et al. (1998) |
| \textit{nat} | Resistance to nourseothricin | No/— | Goldstein & McCusker (1999) |
| \textit{CYH2} | Resistance to cycloheximide | No/— | Delpozo et al. (1991) |
| \textit{pat} | Resistance to bialaphos | No/— | Goldstein & McCusker (1999) |
| \textit{AR04-OPF} | Resistance to o-Fluoro-DL-phenylalanine | No/— | Cebollero & Gonzalez (2004) |
| \textit{SMR1} | Resistance to sulfometuron methyl | No/— | Xie & Jimenez (1996) |
| \textit{FZFI-4} | Increased tolerance to sulfite | No/— | Cebollero & Gonzalez (2004) |
| \textit{DsdA} | Resistance to ω-Serine | No/— | Vorachek-Warren & McCusker (2004) |

Dominant markers

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after marker removal. Commonly, the bacterial sequence hisG is present as direct repeat. However, repeated use of URA3 cassettes carrying hisG increases the probability of mistargeted integrations (Davidson & Schiestl, 2000). An alternative to hisG is the creation of direct repeats upon integration using sequences already present in the host genome, generating seamless marker removal (Akera et al., 2006). Similar to URA3, two other auxotrophic markers MET15 and LYS2 can be counter-selected in the presence of methyl-mercury (Singh & Sherman, 1974) and alpha-aminoadipate (Chattoo et al., 1979), respectively. Nevertheless, their use is limited by the same complications described for URA3.

A successful attempt to recycle virtually any desired marker was the development of the bacteriophage-derived LoxP-Cre recombinase system (Hoes & Abremski, 1985; Sauer, 1987; Guldener et al., 1996, 2002). Exploiting the site-specific activity of the Cre recombinase, this system is used to efficiently remove markers by flanking them with the targeted LoxP sequence. This system exhibits two major limitations: (1) it requires the expression of a plasmid-borne recombinase and thereby necessitates an additional selection marker or extensive screening (Schorsch et al., 2009); and (2) the repeated use of this system causes major chromosomal rearrangements (Dehner et al., 2000; E. Boles, pers. commun.).

The Aspergillus nidulans amdS gene encoding acetamidase has been successfully used as dominant ‘gain of function’ selection marker in different filamentous fungi and the yeast Kluyveromyces lactis (Kelly & Hynes, 1985; Beri & Turner, 1987; Yamashiro et al., 1992; Swinkels et al., 1997; Selten et al., 2000; van Ooyen et al., 2006; Read et al., 2007; Ganatra et al., 2011). Although Selten et al. (2000) suggested that amdS could be used for selection in S. cerevisiae, this statement was not further supported by experimental evidence. Acetamidase catalyzes the hydrolysis of acetamide to acetate and ammonia, thus conferring the ability to the host cell to use acetamide as sole nitrogen or carbon source (Corrick et al., 1987; Hynes, 1994). Similar to URA3, amdS is a recyclable marker that can be counter-selected by growth on media containing the acetamide homologue fluoroacetamide, which is converted by acetamidase to the toxic compound fluoroacetate (Apirion, 1965; Hynes & Pateman, 1970).

This study evaluates the use of the new dominant marker module amdSYM for S. cerevisiae and demonstrates its efficiency for sequentially introducing multiple gene deletions in yeast. Availability of this dominant, counterelectable marker cassette to the yeast research community should facilitate rapid introduction of multiple genetic modifications into any laboratory, wild, and industrial Saccharomyces strains.

Material and methods

Strains and media

Propagation of plasmids was performed in chemically competent Escherichia coli DH5α according to manufacturer instructions (Z-competent transformation kit; Zymo Research, CA). All yeast strains used in this study are listed in Table 2. Under nonselective conditions, yeast was grown in complex medium (YPD) containing 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, and 20 g L⁻¹ glucose. Synthetic media (SM) containing 3 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 5 g L⁻¹ (NH₄)₂SO₄, 1 mL L⁻¹ of a trace element solution as previously described (Verduyn et al., 1992), 1 mL L⁻¹ of a vitamin solution (Verduyn et al., 1992) were used. When amdSYM was used as marker, (NH₄)₂SO₄ was replaced by 0.6 g L⁻¹ acetamide as nitrogen source and 6.6 g L⁻¹ K₂SO₄ to compensate for sulfate (SM-Ac). Recycled markerless cells were selected on SM containing 2.3 g L⁻¹ fluoroacetamide (SM-Fac). SM, SM-Ac, and SM-Fac were supplemented with 20 mg L⁻¹ adenine and 15 mg L⁻¹ L-canavanine sulfate when required. In all experiments, 20 g L⁻¹ of glucose was used as carbon source. The pH in all the media was adjusted to 6.0 with KOH. Solid media were prepared by adding 2% agar to the media described above.

amdSYM and plasmid construction

A codon-optimized [JCat, http://www.jcat.de/, (Grote et al., 2005)] version of A. nidulans amdS flanked by Sall and XhoI restriction sites and attB1 and attB2 recombination sites (Accession number: JX500098) was synthesized at GeneArt AG (Regensburg, Germany) and cloned into the vector pMA (GeneArt AG) generating the plasmid pUD171. The amdS gene was transferred into the destination plasmid pAG426GPD (Alberti et al., 2007) by LR recombination reaction according to the manufacturer recommendations (Invitrogen, CA) yielding the plasmid pUDE158.

The Ashbya gossypii TEF2ter from pUG6 (Guldener et al., 1996, 2002) was amplified, and the restriction sites XhoI and KpnI incorporated with the primers p1FW and p1RV and Phusion Hot Start Polymerase (Finnzymes, Vantaa, Finland). Restriction with XhoI and KpnI and ligation of the amplified fragment and the vector p426TEF (Mumberg et al., 1995) resulted in the replacement of the CYC1ter of p426TEF for (A.g.) TEF2ter. The generated vector was termed p426TEF-TEF(t).

Primers amdBFW and amdSXRV and Phusion Hot Start Polymerase (Finnzymes) were used to amplify amdS and to incorporate BamHI and XhoI sites using pUD171 as
Table 2. Saccharomyces sp. strains used in this study

| Strain   | Genotype                  | References                                |
|----------|---------------------------|-------------------------------------------|
| CEN.PK113-7D | MATa MAL2-8c SUC2         | van Dijken et al. (2000), Entian & Kotter (2007), Nijkamp et al. (2012) |
| CEN.PK113-5D | MATa MAL2-8c SUC2 ura3-52 | van Dijken et al. (2000), Entian & Kotter (2007) |
| CBS8066   | MATa/ho IMK474 CBS12357   | CBS, Den Haag, the Netherlands            |
| IMK473    | Saccharomyces pastorianus | Canelas et al. (2010)                     |
| IMK470    | Saccharomyces cerevisiae  | Mortimer & Johnston (1986)                |
| YSBN      | MATa/ho IMK473 Scottish Ale | Dunn & Sherlock (2008)               |
| S288c     | MATa ho::Blzho::HphMX4 IMK470 derived from CEN.PK113-7D | Gift from Dr. J.M Geertman (Heineken Supply Chain, Zoeterwoude, the Netherlands) |
| CBS1483   | Saccharomyces pastorianus | Libkind et al. (2011)                     |
| CBS12357  | Saccharomyces eubayanus sp.nov | This study                   |
| IME141    | MATa MAL2-8c SUC2 ura3-52 pAG426GPD | This study                   |
| IME142    | MATa MAL2-8c SUC2 ura3-52 pUDE158 | This study                   |
| IMX168    | MATa MAL2-8c SUC2 can1.Δ::amdSYM ADE2 | This study                   |
| IMX200    | MATa MAL2-8c SUC2 can1.Δ ADE2 | This study                   |
| IMX201    | MATa MAL2-8c SUC2 can1.Δ ade2.Δ::amdSYM | This study                   |
| IMX206    | MATa MAL2-8c SUC2 can1.Δ ade2.Δ::amdSYM | This study                   |
| IMK468    | MATa MAL2-8c SUC2 hxk1.Δ::loxP- amdSYM-loxP       | This study                   |
| IMK470    | CBS1483 Sc-hxk1.Δ::loxP- amdSYM-loxP       | This study                   |
| IMK473    | Scottish Ale Sc-aro80.Δ::loxP- amdSYM-loxP       | This study                   |
| IMK474    | CBS12357 Seub-aro80.Δ::loxP- amdSYM-loxP       | This study                   |

Deletion cassette construction

Deletion cassettes were constructed by PCR using Phusion Hot Start Polymerase (Finnzymes) and following manufacturer recommendations. Primers used for repeated gene deletions had a similar design described as follows. Forward primers contain two cores: (1) a 50- to 55-bp sequence homologous to the region upstream the gene to delete and (2) the sequence 5’-CAGCTGAAGCTTCCGTACGC-3’ that binds to the region upstream the A. gossypii TEF2 promoter in pUG-amdSYM. Reverse primers contained three cores: (1) a 50- to 55-bp sequence homologous to the region downstream the gene to be deleted, (2) a 40-bp sequence homologous to the region upstream the targeted region, and (3) the sequence 5’-GCATAGGCCACTAGTGGATCTG-3’ that binds downstream the A. gossypii TEF2 terminator in pUG-amdSYM. CAN1 and ADE2 deletion cassettes were constructed using the primer pairs CdcamdSRV and AdcamdSRV, respectively (Table 3).

To construct the deletion cassette targeting S. cerevisiae HXK1 (Sc.HXK1), primers HdcamdSFW and HdcamdSRV and the template pUG-amdSYM were used. The constructed cassette was used to generate the strain IMK468 derived from CEN.PK113-7D. Genomic DNA of IMK468 was used as template for primers ScHdcamdSFW and ScHdcamdSRV. The resulting cassette contained 500-bp homologous sequences upstream and downstream of the Sc.HXK1 gene and was used for the deletion of Sc.HXK1 in the Saccharomyces pastorianus lager brewing strain CBS1483. The construction of the deletion cassette corre-
sponding to Sc.ARO80 allele for the S. cerevisiae Scottish Ale strain was performed according to the two-step fusion protocol (Amberg et al., 1995) with the primers ScARO80dcamdSFW and ScARO80dcamdSRV to amplify amdSYM from pUG-amdSYM, and ScARO80g5′FW, ScARO80g5′RV, ScARO80g3′FW, and ScARO80g3′RV to generate the 500-bp sequence homologous to upstream and downstream sections of Sc.ARO80, and genomic DNA from CBS1483 was used as template. The same approach was taken for the deletion of Saccharomyces eubayanus ARO80 (Sb.ARO80) in CBS12357 using the primer pairs SeubARO80dcamdSFW/SeubARO80dcamdSRV, SeubARO80g5′FW/SeubARO80g5′RV, and SeubARO80g3′FW/SeubARO80g3′RV. All primers and their sequences are listed in Table 3.

Selection and marker recycling

Yeast transformations were performed using the lithium acetate protocol (Gietz & Woods, 2002). Integration of the deletion cassettes into the yeast genome was selected by plating the transformation mix on SM-Ac. Targeted integration was verified by PCR with the primer pairs CdcFW/CdcRV, AdcFW/AcdRV, HdcFW/HdcRV, ScARO80dcFW/amdSdcRV, ScARO80dcFW/ScARO80gRV, ShARO80dcFW/amdSdcRV, ShARO80dcFW/ShARO80gRV and, when applicable, by transferring single colonies to SM plates containing 15 mg L⁻¹ l-canavanine or by screening for colony pigmentation. A small fraction of single colony was resuspended in 15 μL of 0.02 N NaOH; 2 μL of this cell suspension was used as template for the PCR that was performed using DreamTaq PCR master mix (Fermentas GmbH, St. Leon-Rot, Germany) following the manufacturer recommendations. Marker removal was achieved by growing cells overnight in liquid YPD and transferring 0.2 mL to a shake flask containing 100 mL of SM-Fac. Marker-free single colonies were obtained by plating 0.1 mL of culture on SM-Fac solid media and confirmed by PCR with the primer pairs CdcFW/CdcRV or AdcFW/AcdFW. All cultures were incubated at 30 °C. To confirm that only endogenous sequences were present after marker removal, long run sequencing (Baseclear, Leiden, the Netherlands) was performed using the primers C-FW and C-RV for the CAN1 locus and A-FW and A-RV for ADE2 locus of the marker-free strain IMX206.

Results and discussion

Expression of amdS in S. cerevisiae confers the ability to grow on acetamide as sole nitrogen source

Although the yeast putative amidase gene AMD2 is similar (57.2% similarity and 33.2% identity) to A. nidulans amdS (Chang & Abelson, 1990), there is no report demonstrating acetamidase activity in wild-type S. cerevisiae or on growth of budding yeast on acetamide as sole nitrogen or carbon source. Expression of amdS in S. cerevisiae is therefore expected to bring a new function in this yeast by enabling its growth on acetamide as sole nitrogen source. Although the Aspergillus nidulans amdS promoter is able to drive the expression of genes in S. cerevisiae, this is only possible under carbon-limited conditions (Bonnefoy et al., 1995). Therefore, a codon-optimized amdS sequence was cloned under the control of the strong, constitutive TDH3 promoter in the plasmid pUDE158.

The plasmid pUDE158, containing amdS, and the empty vector pAG426GPD were transformed into CEN.PK113-5D, generating strains IME142 and IME141, respectively. Expression of the acetamidase gene in S. cerevisiae (strain IME142) conferred growth with acetamide as sole nitrogen source, while the control strain (strain IME141) was unable to grow (Fig. 2). Additionally, the inability of IME142 to grow on acetamide upon loss of amdS by counter selection with 5-FOA of pUDE158 confirmed that growth on acetamide was fully amdS dependent (data not shown).
### Table 3. Primers used in this study

| Primer       | Sequence 5′–3′† |
|--------------|-----------------|
| **Plasmid construction** |               |
| amdSBFW      | GGGGGATCCATGCACAATCTTGGAAGAA |
| amdSRV       | GGGCTGAATGTTAGGTAACAG      |
| p1FW         | CGGCTGAATGCTGACAAATGTTAGAG |
| p1RV         | CGGTACCCAGTAAAGCGGCTCAGGTC |
| pUGFW        | GGGGGATCCATGGGTATTCGACAGATG |
| **Deletion cassette construction** |               |
| CdcamdSFW    | TCGAATTCTCTTAATCTCTGTAGAAAA |
| CdcamdSRV    | ATGCCGAATGCGGAATGATATCCAAG |
| AdcamdSFW    | TACTAATACAATAACAACTGGAAGC |
| AdcamdSRV    | TCATTTTATAATATTATCTTGAATCT |
| HdcamdSFW    | AAATCTTACCCAAAATCTCATAAGTC |
| HdcamdSRV    | AGGGAGGAGAAAACAACTTTTATATC |
| ScHdcamdSFW  | CTCTTATGCCCTGAACCC       |
| ScHdcamdSRV  | CTAACCTTACGGTTTCTCCT    |
| Scg5′FW      | AGTTAGTCGTAGGAATATATGAT |
| Scg5′RV      | GGACAACGATACTGAAAGTAAT |
| Scg3′FW      | GCCCTCACATCAAAAAAGAG |
| Scg3′RV      | GTGTGGCTTGTACCACAAGA |
| **Deletion/Marker removal confirmation** |               |
| CdcFW        | CGGAGCAGATGGTGATGCTTG |
| CdcRV        | GGGTTGAGAAAGCAGTATACAA |
| AdFW         | AAGGACATGTGGTACAGGG |
| AdRV         | AGCATTTACGCTGAAATGTTAG |
| HdcFW        | CTGCATAGGCGATTACAGAG |
| HdcRV        | GACGGCAAAAACATACAGG |
| Scg5′FW      | TGGATCCGATACTGGAAATTAC |
| Scg5′RV      | CGACAGCTTACGAGAG |
| Scg3′FW      | GCCCTACATCAAAAAAGAG |
| Scg3′RV      | GTGTGGCTTGTAACAGAA |

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Plasmids and deletion cassettes construction

The coding sequence of the *A. nidulans* *amdS* gene, codon-optimized for expression in *S. cerevisiae* and flanked by the *A. gossypii* TEF2 promoter and terminator, was cloned into the vector pUG6 (Guldener et al., 1996, 2002) by replacing the KanMX gene, resulting in the plasmid pUG-amdSYM (Fig. 1). The resulting *amdSYM* module only contained heterologous sequences, thereby reducing the probability of mistargeted integration (Wach et al., 1994). The pUG-amdSYM plasmid can be easily used as template for deletion cassettes containing the new marker module *amdSYM* and was used for the construction of all deletion cassettes used in this study.

The deletion cassettes contained three major regions (Fig. 3A): (1) a 50- to 55-bp sequence homologous to the upstream part of the gene to be deleted, including the start codon, and a 50- to 55-bp sequence homologous to the downstream part of the gene to be deleted, including the stop codon. These regions were used for targeted homologous recombination (Baudin et al., 1993); (2) the *amdSYM* marker and (3) a 40-bp sequence homologous to the region upstream of the targeted locus to create direct repeats in the host genome upon integration.
thereby enabling scarless marker excision (Akada et al., 2006; Fig. 3).

Repetitive gene deletions in S. cerevisiae using amdSYM

To evaluate whether the new marker amdSYM was suitable for repeated gene knock-out in S. cerevisiae, it was attempted to sequentially delete two genes in the laboratory strain CEN.PK113-7D and, after marker recycling, to construct a marker-free and scarless double-deletion strain. CAN1 and ADE2 were selected for this proof-of-principle experiment because the phenotype caused by CAN1 or ADE2 deletion can be visually screened, giving a fast preliminary evaluation of targeted integration. CAN1 encodes an L-arginine transporter that can also import the toxic compound L-canavanine. Mutants with disrupted CAN1 are able to grow in media containing L-canavanine (Ahmad & Bussey, 1986). ADE2 codes for the enzyme phosphoribosylaminomimidazol carboxylase, which is involved in the biosynthesis of purine nucleotides. ade2 mutants require an external source of adenine and accumulate precursors of purine nucleotides in the vacuole which give colonies a red color (Zonneveld & Vanderzanden, 1995; Fig. 3B).

Fig. 3. Sequential gene deletions methodology. (A) Cassette design for targeted gene deletion and seamless marker removal. (B) Experimental procedure for the sequential deletion of CAN1 and ADE2 in Saccharomyces cerevisiae by amdSYM recycling. (C) Sequencing results of the CAN1 loci of the marker-free IMX206 (can1Δ ade2Δ).
The potential of *amdSYM* as dominant marker was tested by transforming a deletion cassette to disrupt *CAN1* in CEN.PK113-7D. After transformation, cells were grown on synthetic medium (SM) agar plates containing acetamide as sole nitrogen source (SM-Ac, Fig. 4A). Targeted gene deletion was confirmed by the ability of single colonies to grow on SM containing L-canavanine (Fig. 4A) and by PCR (Fig. 4B). The average transformation efficiency was 14 transformants g⁻¹ of DNA, with 100% of the colonies harboring the correct integration. It is important to note that *amdSYM* did not yield false positives.

Although the transformation efficiency reported here for the deletion of *CAN1* may appear low as compared to efficiencies reported for other dominant markers such as Kan-MX (100 transformants g⁻¹ DNA) (Guldener et al., 1996), in our experience, the targeted region and the sequence of the deletion cassette affects much more the transformation efficiency than the nature of the marker used. To support this, it has recently been shown that nucleosome density is a critical factor for transformation efficiency, thereby demonstrating that the localization of a deletion cassette has a strong impact on the transformation efficiency (Aslankoohi et al., 2012). With deletions cassettes using *amdSYM*-based selection but targeted to other loci than *CAN1*, we observed transformation efficiencies similar to those reported for classical selection markers (data not shown).

The resulting strain IMX168 was subsequently used to evaluate the potential of marker excision aided by the direct repeats created upon integration (Fig. 3B). Under nonselective conditions, that is, growth in complex media (YPD), mitotic recombination between the direct repeats flanking *amdSYM* may excise the marker. To select for these recombinants, strain IMX168 (*can1Δ::amdS*) was grown overnight in liquid complex media, transferred to synthetic medium containing fluoroacetamide (SM-Fac), and then plated on SM-Fac. PCR analysis of the growing colonies confirmed correct marker removal (Fig. 4B); the new strain was named IMX200 (*can1A*). Due to the absence of *CAN1*, IMX200 (*can1A*) was able to grow on media containing L-canavanine. As anticipated, it had lost the ability to grow on media containing acetamide as

![Fig. 4](https://academic.oup.com/femsyr/article-abstract/13/1/126/544401/fig4)

**Fig. 4.** Sequential gene deletions of *CAN1* and *ADE2* using *amdSYM* in *S. cerevisiae*. (A) The strains IMX168, IMX200, and IMX201 and the parental strain CEN.PK113-7D were grown on SM-Ac, SM-Ac supplemented with adenine, and SM supplemented with adenine and L-canavanine. The plates were incubated at 30 °C and were read after 3 days. (B) PCR analysis to confirm correct integration of the gene disruption cassettes and their removal at the *CAN1* and *ADE2* loci. PCR was carried out on reference CEN.PK113-7D, IMX168, IMX200, and IMX201. All PCRs were performed with the primer pairs CdcFW/CdcRV and AdcFW/AdcRV for *CAN1* and *ADE2* loci, respectively. In the parental strain, amplification of the *CAN1* and *ADE2* loci generated fragments of 2151 bp (a) and 1950 bp (d) for *CAN1* and *ADE2*, respectively. PCR on IMX168 DNA generated a fragment of 2835 bp (b) due to the incorporation of *amdSYM* in the *CAN1* locus. A short fragment of 333 bp (c) was obtained for IMX200 as a result of *amdSYM* excision from the *CAN1* locus. Similarly, the disruption of *ADE2* using *amdSYM* led to a large PCR product of 2691 bp in IMX201 (e) while PCR on the *ADE2* locus in the marker-free strain IMX206 generated a short fragment of 189 bp (f). The products obtained were then subjected to agarose gel electrophoresis.
nucleotide sequence, was a dominant selectable marker in the prototrophic laboratory strain CEN.PK113-7D and that the marker can be removed to avoid protein burden. An important addition is that the design of the deletion cassette allows for marker removal leaving only endogenous sequences (Fig. 3C).

A strong feature of recyclable markers is that a single marker is sufficient to perform multiple sequential manipulations in the same strain. The potential of amdSYM for serial deletion was tested by deleting a second gene in the marker-free strain IMX200 (can1Δ). After transformation with an amdSYM deletion cassette targeted to ADE2 locus, correct transformants could be easily selected for their ability to grow in the presence of L-canavanine and to use acetamide as nitrogen source and for their auxotrophy for adenine and red pigmentation (Fig. 4A). The strain IMX201 (can1Δ ade2Δ::amdSYM) was selected using these criteria, and correct integration of the deletion cassette was confirmed by PCR (Fig. 4B). This second deletion demonstrated that amdSYM is a powerful selection marker for serial gene deletion. IMX201 was further engineered to generate the marker-free strain IMX206 (can1Δ ade2Δ) by scarless removal of amdSYM. This was confirmed by sequencing the CAN1 and ADE2 loci in IMX206. Similar to the parental strain CEN.PK113-7D, strain IMX206 (can1Δ ade2Δ) was unable to grow on media containing acetamide as sole nitrogen source but, due to the deletions performed, showed a red pigmentation, was not able to grow in absence of adenine source but, due to the deletions performed, showed a red pigmentation (Fig. 4A). The strain IMX201 (can1Δ ade2Δ::amdSYM) was selected using these criteria, and correct integration of the deletion cassette was confirmed by PCR (Fig. 4B). This second deletion demonstrated that amdSYM is a powerful selection marker for serial gene deletion. IMX201 was further engineered to generate the marker-free strain IMX206 (can1Δ ade2Δ) by scarless removal of amdSYM. This was confirmed by sequencing the CAN1 and ADE2 loci in IMX206. Similar to the parental strain CEN.PK113-7D, strain IMX206 (can1Δ ade2Δ) was unable to grow on media containing acetamide as sole nitrogen source but, due to the deletions performed, showed a red pigmentation, was not able to grow in absence of adenine, and was able to grow on media containing l-canavanine (Fig. 4). This marker- and scar-free strain can be subsequently used for additional deletions or other genetic manipulations.

The module amdSYM can be used in a wide range of laboratory, wild, and industrial Saccharomyces strains

The first condition to use amdSYM as selectable marker is that the parental strain does not have the capability to use acetamide as sole nitrogen source. To verify whether other laboratory strains besides the strains of the CEN.PK lineage could be modified using amdSYM as marker, the ability to grow on media containing acetamide as nitrogen source of three popular laboratory strains, namely CBS8066, YSBN [i.e. a prototrophic BY strain (Canelas et al., 2010)], and S288c (Mortimer & Johnston, 1986), was tested. None of the laboratory strains were able to use acetamide as sole nitrogen source (Fig. 5). To further expand the range of species in which amdSYM could be used, two brewing strains, the S. cerevisiae Scottish Ale strain and the S. pastorianus lager brewing strain CBS1483, and the wild yeast S. eubayanus CBS12357 were tested for their ability to grow on acetamide as sole nitrogen source. Similarly to the laboratory strains, none of these Saccharomyces species grew with acetamide as sole nitrogen source (Fig. 6A). The absence of endogenous acetamidase activity demonstrated that amdSYM could potentially be used as selectable marker in a wide range of laboratory, wild, and industrial Saccharomyces strains.

To confirm the universality of amdSYM as selectable marker, genes were deleted using amdSYM in the above-mentioned industrial and wild yeast strains. To compensate for the expected lower efficiency of homologous recombination in these non-cerevisiae species, the deletion cassettes were designed with longer homologous flanking regions of at least 500 bp. The gene HXK1 was deleted in both the laboratory strain CEN.PK113-7D and the S. pastorianus lager brewing strain CBS1483 using cassettes containing amdSYM. Saccharomyces pastorianus is a hybrid species that contains two subgenomes: one that resembles S. cerevisiae genome and another one similar to S. eubayanus (Libkind et al., 2011). In this study, only the S. cerevisiae allele (Sc.HXK1) was deleted. The strains generated were named IMK468 (Sc.hxk1Δ::amdSYM) for the CEN.PK mutant and IMK470 (Sc.hxk1Δ::amdSYM) for the S. pastorianus mutant. Additionally, the S. cerevisiae Scottish Ale brewing strain and the recently described wild yeast S. eubayanus CBS12357 were also genetically modified using amdSYM. Making use of amdSYM as marker cassette the gene Sh.ARO80 was deleted in S. eubayanus resulting in the strain IMK474, and Sc.ARO80 was deleted in the S. cerevisiae Scottish Ale strain, generating the strain IMK473.

IMK468, IMK470, IMK473, and IMK474 all demonstrated the integration of amdSYM in their genomes by their ability to grow on acetamide as sole nitrogen source.
PCR analysis confirmed the deletion of the targeted genes (Fig. 6B). The growth on acetamide plates was slower for industrial strains as compared to CEN.PK-derived deletion mutants, but increasing the acetamide concentration resulted in faster growth, thereby accelerating the screening process (data not shown). Therefore, the amount of acetamide necessary for amdSYM-based strain construction may be strain-dependent and requires optimization.

**Conclusions**

When transformed into yeast, *A. nidulans* amdS conferred the capability to use acetamide as sole nitrogen source. This gain of function allowed the use of amdS as a new dominant marker in *S. cerevisiae*. In the present work, a new heterologous module, amdSYM, which encompasses the regulatory regions of *A. gossypii* TEF2 and the *A. nidulans* gene amdS, was constructed and has been made available for the research community in the widely used plasmid series as pUG-amdSYM. Not only is amdSYM a new dominant marker, but it is also an additional counter-selectable marker in the yeast genetic toolbox. A strong feature of amdSYM is that, contrary to all other counter-selectable markers available, it does not require a specific genetic background for the strain to be selected.

Furthermore, while most marker recycling methods, such as the LoxP-Cre recombinase system, have the disadvantage of leaving scars after each recycling, in the present study, marker removal was scarless (Akada *et al.*,...
Saccharomyces opens the door to fast and easy genetic manipulation in expected to considerably contribute to the functional and hybrid genome of newly discovered wild species of fully used as selection marker to perform deletions in the cerevisiae MET15 expression of several heterologous markers.

In conclusion, the new marker module amdSYM is an excellent tool for consecutive genetic modifications in the yeast S. cerevisiae and a good alternative to URA3, MET15, or LYS2 with the additional substantial advantage that it is not limited to specific strain backgrounds or to the cerevisiae species. amdSYM has indeed been successfully used as selection marker to perform deletions in the newly discovered wild species of S. eubayanus and in S. cerevisiae and S. pastorianus brewing strains. Thanks to this later success in deleting genes from the aneuploid and hybrid genome of S. pastorianus, amdSYM is expected to considerably contribute to the functional analysis of genes in strains with complex genome architecture. As the first dominant recyclable marker, amdSYM opens the door to fast and easy genetic manipulation in Saccharomyces laboratory, wild, and industrial strains.

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