New cassettes for single-step drug-resistance and prototrophic marker switching in fission yeast

Alexander Lorenz

Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK
Correspondence to Alexander Lorenz (Phone: +44 1224 437323, Fax: +44 1224 437465, e-mail: a.lorenz@abdn.ac.uk)

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Abstract Construction of multiply mutated strains for genetic interaction analysis and of strains carrying different epitope tags at multiple open reading frames for testing protein localization, abundance and protein-protein interactions is hampered by the availability of a sufficient number of different selectable markers. Moreover, strains with single gene deletions or tags often already exist in strain collections; for historical reasons these will mostly carry the *ura4*$^+$ gene or the G418-resistance *kanMX* as marker. Because it is rather cumbersome to produce multiply deleted or tagged strains using the same marker or to completely reconstruct a particular strain with a different marker, single-step exchange protocols of markers are a time-saving alternative. In recent years dominant drug resistance markers (DDRMs) against clonNAT, Hygromycin B, and Bleomycin have been adapted and successfully used in *Schizosaccharomyces pombe*. The corresponding DDRM cassettes – *natMX*, *hphMX*, and *bleMX* – all carry the *TEF*-promotor and -terminator sequences from *Ashbya gossypii* as *kanMX*, this provides flanking homologies to enable single-step marker swapping by homologous gene targeting. To expand this very useful toolset for single-step marker exchange I constructed *MX*-cassettes containing the nutritional markers *arg3*$^+$, *his3*$^+$, *leu1*$^+$, and *ura4*$^+$. Furthermore, a set of constructs was created to enable single-step exchange of *ura4*$^+$ to *kanMX6*, *natMX4*, and *hphMX4*. The functionality of the cassettes is demonstrated by successful single-step marker swapping at several loci. These constructs allow a straight-forward and rapid re-marking of existing *ura4*$^+$- and *MX*-deleted and -tagged strains.

Key words *Schizosaccharomyces pombe*, selectable marker, marker switch, plasmid, PCR

Introduction

As the field of molecular biology using the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as model organisms matured and moved into a postgenomic era, collections of strains with deletions or tagged versions of genes have been created. These collections originated either from a concerted effort to create genome-wide sets of gene deletions or tags [Hayashi et al. 2009; Kim et al. 2010; Spirek et al. 2010], or from the realization that availability of yeast strains beyond the life-time of a particular research group would be hugely beneficial to the scientific community requiring long-term storage in bio-repositories such as the National BioResource Project (NBRP) of the MEXT, Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html).

Historically, the most widely used selectable markers in *Sz. pombe* are the *ura4*$^+$ gene and *kanMX6*, which confers resistance to the antibiotic G418 (geneticin) [Grimm et al. 1988; Bähler et al. 1998]; the *S. cerevisiae LEU2*$^+$ gene can rescue *Sz. pombe leu1* mutants when provided in multiple copies, which made it the ideal choice for a genetic marker on plasmid vectors [Beach and Nurse 1981; Maundrell 1993]. This rather small selection of markers is a potential hindrance when studying complex genetic interactions involving several factors and when investigating the subcellular localization of multiple proteins, or protein-protein interactions. To overcome this problem additional selectable markers have been introduced to *Sz. pombe*, these include genes conferring resistance to the antibiotics clonNAT (nourseothricin), Hygromycin B, Bleomycin/Phleomycin, and Blasticidin S [Rabitsch et al. 2004; Sato et al. 2005;

Hentges et al. 2005; Gregan et al. 2006; Erler et al. 2006]. The *natMX* (resistance against clonNAT), *hphMX* (resistance against Hygromycin B), and *bleMX* (resistance against Bleomycin/Phleomycin) constructs made use of the same promoter and terminator sequences from the *TEF* gene of the filamentous ascomycete *Ashbya gossypii* as the original *kanMX* cassette [Wach et al. 1994; Bähler et al. 1998; Goldstein and McCusker 1999; Sato et al. 2005; Hentges et al. 2005]. Especially, the *natMX* and *hphMX* (sometimes also referred to as *hygMX*) cassettes have been widely adopted to create multiply tagged and/or deleted strains [e.g., Rabitsch et al. 2004; Masuda et al. 2013; Ding and Forsburg 2014; Lorenz et al. 2014; Okada et al. 2014; Tsang et al. 2014]. The *TEF* sequences provide flanking homologies of a few 100 nucleotides which enable a simple single-step switching of selectable markers via targeted homologous integration [Sato et al. 2005; Hentges et al. 2005].

The constructs presented and validated here simplify the construction of multiply deleted and/or tagged fission yeast strains from already existing genetic modifications. This serves a different purpose than previously described 'marker switch' and 'knock-in' approaches for the targeted introduction of mutated open reading frames into wild-type cells [MacIver et al. 2003; Mudge et al. 2012; Fennessy et al. 2014]. The constructs described here offer new opportunities by enabling the direct exchange of *ura4*$^{+}$ to DDRMs (dominant drug resistance markers) and of *MX*-type antibiotic markers to prototrophic markers in a simple, single-step transformation protocol.

Materials and Methods

Enzymatic reactions for plasmid constructions and PCR amplification of DNA fragments for transformation

PCR reactions for plasmid constructions were performed using either Phusion (Thermo Fisher Scientific Inc., Waltham, MA, USA) or Q5 (New England BioLabs Inc., Ipswich, MA, USA) high-fidelity DNA polymerases. Oligonucleotides were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA); and restriction endonucleases, and T4 DNA ligase were obtained from New England BioLabs Inc.. All relevant plasmid sections were verified by DNA sequencing.

The *ura4*$^{+}$ marker swap cassettes were amplified from 100 ng of plasmid DNA (pALo120, pALo121, or pALo122) in a 50 µl PCR reaction (10 µl 5× Q5 Reaction Buffer, 200 µM dNTPs, 500 nM each of AL1forw and AL1rev oligonucleotides as primers, and 1 U Q5 high-fidelity DNA polymerase) using the following PCR programme: 30 sec at 98°C – 35× (10 sec at 98°C, 20 sec at 55°C, 85 sec at 72°C) – 120 sec at 72°C (Figure 1B, Table 1). The *MX* marker swap cassettes were amplified from 100 ng of plasmid DNA (pFA6a-*arg3MX4*, pFA6a-*his3MX4*, pFA6a-*leu1MX4*, or pFA6a-*ura4MX4*) in a 50 µl PCR reaction (10 µl 5× Q5 Reaction Buffer, 200 µM dNTPs, 500 nM each of AL2forw and AL2rev oligonucleotides as primers, and 1 U Q5 high-fidelity DNA polymerase) using the following PCR programme: 30 sec at 98°C - 35× (10 sec at 98°C, 20 sec at 64°C, 90 sec at 72°C) – 120 sec at 72°C (Figure 2B, Table 1).

Growth, media, and transformation

For growing *E. coli* LB and SOC media – where appropriate containing 100 µg/ml Ampicillin – were used [Sambrook and Russell 2000]. Competent cells from *E. coli* strains NEB5-alpha, NEB10-beta (New England BioLabs Inc.), Stellar (Clontech Laboratories Inc.), and XL1-blue (Agilent Technologies, Santa Clara, CA) were transformed following the protocols provided by the corresponding manufacturers.

Fission yeast strains used in this study are listed in Table 2. *Schizosaccharomyces pombe* cells were cultured on yeast extract (YE), and on yeast nitrogen base glutamate (YNG) agar plates containing the required supplements (concentration 250 µg/ml on YE, and 75 µg/ml on YNG) [Sabatinos and Forsburg 2010; Smith 2009]. Concentrations for antibiotics used were: G-418 disulfate (ForMedium, Norfolk, UK) at 200 µg/ml, clonNAT (Werner BioAgents, Jena, Germany) at 200 µg/ml, and Hygromycin B (ForMedium) at 400 µg/ml. All yeast transformations were performed using 1-5 µg of DNA in a standard Li-acetate protocol [Keeney and Boeke 1994], with the following modifications: (I) cells were grown in fully supplemented YE broth, (II) incubation of competent cells at 30°C was done for approximately 4 hours instead of 30-45 minutes, and (III) after the heat shock cells in transformation mix (40% PEG in Li-acetate/TE) were immediately plated onto non-selective fully supplemented YE, and not pelleted and resuspended in water. The

latter two modifications were suggested by Charles Hoffman (Boston College, Chestnut Hill, MA), and strongly improve transformation efficiency (http://listserver.ebi.ac.uk/pipermail/pombelist/2014/004012.html). Yeast cells were grown for 18-24 hours at 30°C on non-selective YE plates, and then replica-plated onto selective media; either fully supplemented YE agar containing an antibiotic (concentrations as above) or supplemented YNG agar lacking the appropriate amino acid or nucleobase.

Obtaining the single-step marker switch cassettes

Plasmids carrying the single-step marker switch cassettes described here will be available from the National BioResource Project (NBRP) of the MEXT, Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html) (Table 3). Plasmid sequences are available online as *.txt-files at http://dx.doi.org/10.6084/m9.figshare.1468419 [Lorenz 2015].

Results and Discussion

Construction of plasmids for marker switching *ura4* to dominant drug resistance markers (DDRMs)

A simple, high-copy vector with a single XbaI-site was created by PCR-amplifying pUC8 [Messing and Vieira 1982] using oligonucleotides AL3forw and AL3rev (Table 1). The resulting PCR product was digested with XbaI, treated with DpnI to remove pUC8 template DNA, and ligated using T4 DNA Ligase. This resulted in pUC8xba (Table 3), in which the entire multi-cloning site of pUC8 is replaced with a single XbaI-site. The *ura4*$^+$ marker [Grimm et al. 1988] was PCR-amplified from pREP42 [Basi et al. 1993] (oligonucleotides AL4forw and AL4rev; Table 1) exchanging the flanking HindIII-sites for XbaI-sites, this PCR product was then cloned into the XbaI-site of pUC8xba. The resulting plasmid was amplified using PCR (oligonucleotides AL5forw and AL5rev; Table 1) to give a fragment containing 5' and 3' *ura4*+-flanking sequences and the pUC8 vector backbone. This DNA fragment was then merged with a *natMX4*-cassette – released from pAG25 [Goldstein and McCusker 1999] by a PvuII-SpeI digestion – using In-Fusion cloning (Clontech Laboratories Inc., CA, USA). Subsequently, the *natMX4* marker was replaced by *kanMX6*- and *hphMX4*-cassettes from pFA6a-*kanMX6* [Bähler et al. 1998] and pAG32 [Goldstein and McCusker 1999], respectively, by standard cloning using BglII- and EcoRV-sites. The resulting plasmids pALo120 (*kanMX6*), pALo121 (*natMX4*), and pAlo122 (*hphMX4*) (Table 3) carry ~220 bp of the 5' and 3' ends of the 1.8kb-fragment of *ura4*$^+$ [Grimm et al. 1988]. These *ura4*$^+$-marker switch cassettes can be liberated by a XbaI restriction digest or PCR using oligonucleotides AL1forw and AL1rev (Figure 1, Table 1). The *ura4*$^+$ gene at its original locus in strain UoA66 was knocked out using PCR products, AL1forw-AL1rev on pALo120, pALo121, and pALo122 (as described above). Correct integration was monitored by testing for an antibiotic-resistant Ura$^-$ phenotype, and frequencies of correct integration between 22.9% and 37.1% were observed (Table 4). Furthermore, three independent *ura4*$^+$-marked constructs *pms1*$^-$::*ura4*$^+$ [Schär et al. 1997], *swi4*Δ::*ura4*$^+$ [Fleck et al. 1992], and *meu13*Δ::*ura4*$^+$ [Nabeshima et al. 2001] were efficiently replaced with the clonNAT (XbaI-digest of pALo121) or Hygromycin B (XbaI-digest of pALo122) resistance gene. Frequencies of correct integration for *pms1*$^-$::*ura4*$^+$ to *pms1-16*::*natMX4* was 40.6% (n = 32), for *swi4*Δ::*ura4*$^+$ to *swi4*Δ-*36*::*hphMX4* was 100% (n = 6), and for *meu13*Δ::*ura4*$^+$ to *meu13*Δ-*22*::*hphMX4* was 93.8% (n= 32). In these instances correct integration was not only tested by an antibiotic-resistant Ura$^-$ phenotype, but also verified by colony PCR on a few selected transformants (data not shown). A slightly different construct with larger *ura4*$^+$-flanking homologies was successfully used to swap a *dmc1*Δ::*ura4*$^+$ to *dmc1*Δ-*12*::*natMX4* [Grishchuk and Kohli 2003; Lorenz et al. 2012]. Frequencies of correct integration are similar to what was reported previously for fission yeast [Bähler et al. 1998; Sato et al. 2005].

Construction of plasmids for targeting the *ura4* open reading frame

The *ura4* gene represents a negatively and positively selectable marker; *ura4*$^+$ cells can grow on minimal media lacking uracil, but are killed by 5-FOA (5-fluoroorotic acid) treatment [Boeke et al. 1984; Grimm et al. 1988]. To fully exploit the dual selection feature of the *ura4* marker, plasmids solely carrying the *ura4*-targeting sequences were also constructed. 5' phosphorylated oligonucleotides AL6forw and AL6rev (Table 1) were used to perform PCR on pALo120. The

resulting PCR product was ligated with T4 DNA ligase after DpnI treatment to remove pALo120 template DNA to give pALo125. This plasmid can be used to remove *ura4*$^+$ for marker recycling, although it should be kept in mind, that this will leave a 440bp scar which still can be targeted by the 1.8kb *ura4*$^+$-fragment [Grimm et al. 1988]. In principle, this is not an issue for constructing multiply deleted or tagged strains with these cassettes, because the initial marker swap will be performed in a background with a single deletion or tag, subsequently differently marked mutants and tags will be combined by crossing.

In order to enable delivering potentially any construct or open reading frame to the *ura4* locus, pALo120 was amplified by PCR using oligonucleotides AL7forw and AL7rev (Table 1). The resulting PCR product was digested with BglII and DpnI, and ligated using T4 DNA ligase, this created a plasmid (pALo126; Table 3) with a large multicloning site (HindIII – BsiWI – PstI – SalI – BamHI – SmaI – PacI – BssHII – BglII – SacI – EcoRI – BspDI – EcoRV – SpeI) flanked by the two ~220bp *ura4*-targeting sequences. The *ura4*$^+$ targeting cassettes can be released from pALo125 or pALo126, respectively, by a XbaI restriction digest or amplified by PCR using oligonucleotides AL1forw and AL1rev (Table 1).

Construction of *arg3MX4*, *his3MX4*, *leu1MX4*, and *ura4MX4* plasmids

To enable marker switching between any DDRM – flanked by *TEF*-promotor and -terminator sequences present in all the *MX* constructs – and the prototrophic markers *arg3*$^+$, *his3*$^+$, *leu1*$^+$, and *ura4*$^+$ pAG25 was amplified using oligonucleotides AL8forw and AL8rev (Table 1). The resulting PCR product was digested with NheI and DpnI, and ligated using T4 DNA ligase. This created pALo123 (Table 3), which carries the *TEF*-promotor and -terminator sequences with an intervening NheI site. The *arg3*$^+$ open reading frame [Waddell and Jenkins 1995] including its own promoter and terminator was amplified by PCR with oligonucleotides AL9forw and AL9rev (Table 1) using genomic DNA from UoA474 as a template, the PCR product was digested with XbaI and cloned into the compatible NheI site of pALo123 resulting in pFA6a-*arg3MX4* (Table 3). The *his3*$^+$ marker was amplified by PCR from pFOX2 [Osman et al. 2000] using oligonucleotides AL10forw and AL10rev (Table 1) replacing the original BglII sites with NheI sites. The resulting PCR product was cloned into the NheI site of pALo123, which resulted in pFA6a-*his3MX4* (Table 3). A functional *leu1*$^+$ open reading frame [Kikuchi et al. 1988] including its own promoter and terminator was amplified by PCR using oligonucleotides AL11forw and AL11rev (Table 1) and genomic DNA from UoA474 as a template. The PCR product was digested with XbaI and cloned into the compatible NheI-site of pALo123 to give pFA6a-*leu1MX4* (Table 3). Finally, the *ura4*$^+$ marker was cloned as an XbaI fragment (see above) into the compatible NheI site of pALo123 to give pFA6a-*ura4MX4* (Table 3). Prototrophic markers are transcribed using their respective original promoters and terminators, P_{TEF}- and T_{TEF}-sequences just provide flanking homology for gene targeting in these constructs.

To perform marker switching with the resulting prototrophic *MX* cassettes, they can be amplified by PCR using oligonucleotides AL2forw and AL2rev (Figure 2, Table 1). Alternatively, they can also be released by a BamHI-EcoRI double restriction digest, except for *his3MX4* which requires a BglII-SacI restriction digest. The *arg3MX4*, *his3MX4*, *leu1MX4*, and *ura4MX4* cassettes were amplified by PCR (as above) from their respective plasmids and transformed into the UoA193 strain, which has the *hop1* gene knocked-out with *kanMX6*. *hop1* codes for a meiosis-specific chromosome axis protein [Lorenz et al. 2004; Lorenz et al. 2006]. Meiosis-specific factors seem to be more refractory to correct targeting than genes coding for non-meiotic proteins [Bähler et al. 1998], therefore the *hop1* locus presents an optimal target to test the efficiency limits of one-step marker swapping. Frequencies of correct integration ranging from 15.3% to 36.6% (Table 4) were observed by monitoring for Arg$^+$ G418-sensitive, His$^+$ G418-sensitive, Leu$^+$ G418-sensitive, and Ura$^+$ G418-sensitive phenotypes.

Conclusion

Here, 7 new constructs for single-step marker replacement are reported and tested for functionality. Three of these will be useful tools for the fission yeast community to swap deletions and tagged versions of open reading frames from the widely-used *ura4*$^+$ marker to 3 antibiotic markers (a.k.a. DDRMs) conferring resistance to G418, clonNAT, or Hygromycin B, respectively. The other 4 can be used to exchange any *MX*-type marker

(flanked by P_{TEF}- and T_{TEF}-sequences) for the *Sz. pombe* genes encoding the prototrophic markers Ura4, Arg3, His3, or Leu1. These marker swap cassettes will simplify the construction of strains deleted and/or tagged at multiple loci.

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Table 1. List of oligonucleotides used

Oligonucleotide No.	Oligo (in 5'→3' direction)
AL1forw	agctacaaatcccactgg
AL1rev	gtgatattgacgaaacttttg
AL2forw	gtttagcttgcctcgtccc
AL2rev	gatggcggcgttagtatcg
AL3forw	aattaaTCTAGAcgtaatcatggtcatagctgtttcc
AL3rev	aattaaTCTAGAggcactggccgtcgttttac
AL4forw	ctctTCTAGAagctacaaatcccac
AL4rev	ctctTCTAGAgtgatattgacgaaac
AL5forw	GATGATATCAGATCCACTAGTcatgctcctacaacattacc
AL5rev	AGCGTACGAAGCTTCAGCTGgctgattatctttttcaccatgc
AL6forw	[phos]gctgattatctttttcaccatgc
AL6rev	[phos]catgctcctacaacattacc
AL7forw	gggacgaggcaagctaaac
AL7rev	aattaaAGATCTgagctcgaattcatcgatg
AL8forw	aattaaGCTAGCcatggttgtttatgttcg
AL8rev	aattaaGCTAGCccctaatcagtactgac
AL9forw	aattaaTCTAGAcgtactagcttgtttgc
AL9rev	aattaaTCTAGAggaagacaagaaaaagcc
AL10forw	aattaaGCTAGCtctatgcaaagctaacgaatc
AL10rev	aattaaGCTAGCtttcaacgttttctttactattgc
AL11forw	aattaaTCTAGAtcgatatcccaatctgtag
AL11rev	aattaaTCTAGAttatgttacaggttacttcg

Table 2. Yeast strain list

Strain	Relevant genotype	Origin
UoA66	*h-smt0 arg3-D4 his3-D1 leu1-32*	lab strain
PRS301[a]	*h- pms1-::ura4+ ura4-D18*	[Schär et al. 1997]
UoA372	*h+S pms1-::ura4+ arg3-D4 his3-D1 leu1-32 ura4-D18*	this study[b]
UoA386	*h+S pms1-16::natMX4 arg3-D4 his3-D1 leu1-32 ura4-D18*	this study
OL278[c]	*h+ swi4Δ::ura4+ ade6-M387 ura4-D18*[d]	[Fleck et al. 1992]
UoA375	*h-smt0 swi4Δ::ura4+ arg3-D4 his3-D1 leu1-32 ura4-D18*[d]	this study[e]
UoA389	*h-smt0 swi4Δ-36::hphMX4 arg3-D4 his3-D1 leu1-32 ura4-D18*[d]	this study
118-4682[f]	*h- meu13Δ::ura4+ ade6-M210 leu1-32 ura4-D18*	[Nabeshima et al. 2001]
UoA581	*h- meu13Δ-22::hphMX4 ade6-M210 leu1-32 ura4-D18*	this study
UoA474	*h- ade6-149*	lab strain
UoA193	*h- hop1Δ-1::kanMX6 arg3-D4 his3-D1 leu1-32 ura4-D18*	lab strain

[a]provided as FY18790 by the National BioResource Project (NBRP) of the MEXT, Japan.
[b]Derivative of PRS301.
[c]provided as FY18812 by the National BioResource Project (NBRP) of the MEXT, Japan.
[d]The original construct as described by Fleck and coworkers [Fleck et al. 1992] also partially deletes the neighbouring open reading frame *dph3*, so these strains in effect are *swi4Δ dph3Δ* double mutants.
[e]Derivative of OL278.
[f]provided by Jürg Kohli.

Table 3. List of plasmids created in this study

Plasmid	FYP number[a]	Insert	Purpose
pALo120	2884	*ura4* 5' flanking sequence – *kanMX6* – *ura4* 3' flanking sequence	*ura4+* to *kanMX6* marker swap
pALo121	2885	*ura4* 5' flanking sequence – *natMX4* – *ura4* 3' flanking sequence	*ura4+* to *natMX4* marker swap
pALo122	2886	*ura4* 5' flanking sequence – *hphMX4* – *ura4* 3' flanking sequence	*ura4+* to *hphMX4* marker swap
pALo123	2887	P_{TEF} – NheI – T_{TEF}	*MX* targeting
pALo125	2888	*ura4* 5' flanking sequence – *ura4* 3' flanking sequence	*ura4+* deletion/marker recycling
pALo126	2889	*ura4* 5' flanking sequence – *MCS* – *ura4* 3' flanking sequence	*ura4+* targeting
pFA6a-*arg3MX4*	2890	P_{TEF} – *arg3+* – T_{TEF}	*MX* to *arg3+* marker swap
pFA6a-*his3MX4*	2891	P_{TEF} – *his3+* – T_{TEF}	*MX* to *his3+* marker swap
pFA6a-*leu1MX4*	2892	P_{TEF} – *leu1+* – T_{TEF}	*MX* to *leu1+* marker swap
pFA6a-*ura4MX4*	2893	P_{TEF} – *ura4+* – T_{TEF}	*MX* to *ura4+* marker swap
pUC8xba	2894	none	general cloning

[a]refer to FYP numbers when obtaining plasmids from the National BioResource Project (NBRP) of the MEXT, Japan.

Table 4. Efficiency of correct integration during marker swap

Strain transformed	Original Marker/Gene	New Marker	Positives/Total	% correct integration
UoA66	*ura4+*	*kanMX6*	8/35	22.9%
UoA66	*ura4+*	*natMX4*	13/35	37.1%
UoA66	*ura4+*	*hphMX4*	8/35	22.9%
UoA193	*kanMX6*	*arg3+*	23/69	33.3%
UoA193	*kanMX6*	*his3+*	11/72	15.3%
UoA193	*kanMX6*	*leu1+*	16/70	22.9%
UoA193	*kanMX6*	*ura4+*	26/71	36.6%

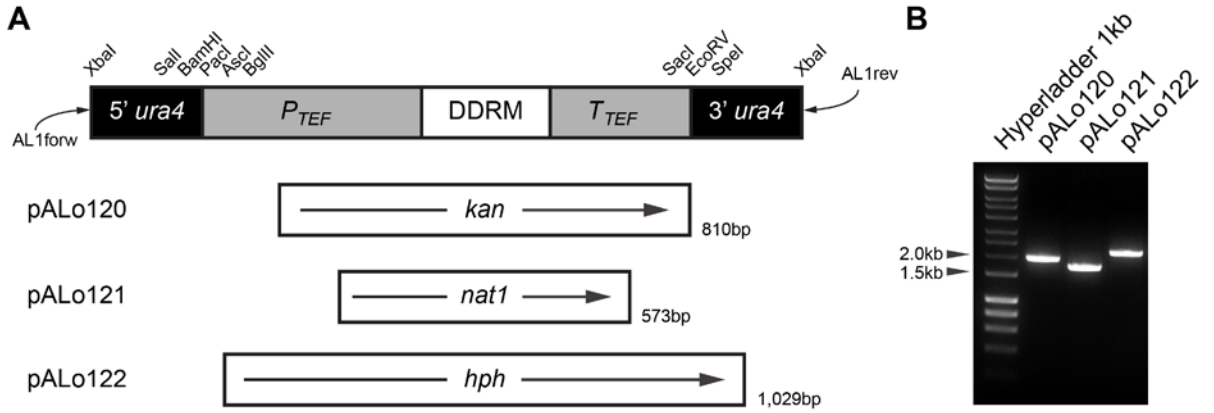

Figure 1. *ura4*⁺ to dominant drug resistance marker (DDRM) swap cassettes. (**A**) Schematic representation of the structure of the *ura4*⁺ gene to DDRM swap cassettes on the corresponding plasmids (pALo120, pALo121, pALo122). Size of the DDRM gene in bp is indicated. The direction of transcription is shown as arrows within the box representing the marker, DDRMs are driven by the *TEF*-promoter (P_{TEF}) and terminated at the *TEF*-terminator (T_{TEF}). The positions of the XbaI sites for cassette release are indicated, as are restriction sites unique in all 3 constructs, for further restriction sites consult the corresponding sequence files. (**B**) Amplifying *ura4*⁺ to DDRM swap cassettes. DNA molecular weight marker in 1st lane is Hyperladder 1kb (Bioline, London, UK) (4 µl). Bands are 2 µl of a 50 µl PCR reaction as described in Materials and Methods. Expected band size for each PCR product as follows: pALo120 (1,973 bp), pALo121 (1,739 bp), and pALo122 (2,195 bp). DNA was loaded onto a 0.8% Agarose (Bioline) gel (1× TAE) containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich Co.).

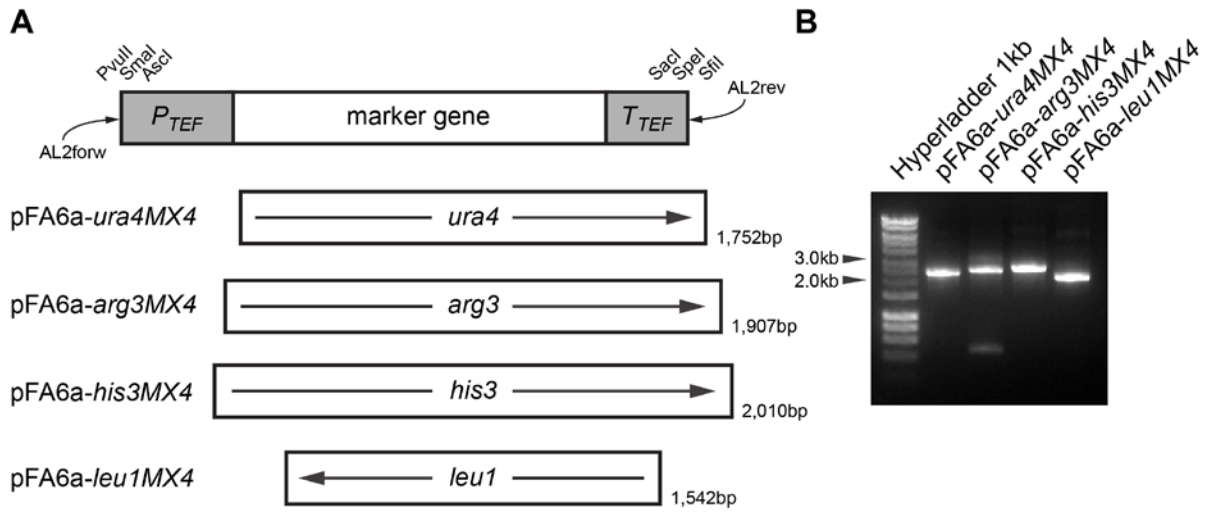

Figure 2. *MX* to prototrophic marker swap cassettes. (**A**) Schematic representation of the structure of the *MX* to prototrophic marker swap cassettes on the corresponding plasmids (pFA6a-*ura4MX4*, pFA6a-*arg3MX4*, pFA6a-*his3MX4*, pFA6a-*leu1MX4*). Size of the corresponding gene in bp is indicated. The direction of transcription is shown as arrows within the box representing the marker, prototrophic markers are transcribed using their respective original promoters and terminators, P_{TEF}- and T_{TEF}-sequences just provide flanking homology for gene targeting in these constructs. Restriction sites unique in all 3 constructs are given, for further restriction sites consult the corresponding sequence files. (**B**) Amplifying *MX* marker swap cassettes. DNA molecular weight marker in 1st lane is Hyperladder 1kb (Bioline) (4 µl). Bands are 2 µl of a 50 µl PCR reaction as described in Materials and Methods. Expected band size for each PCR product as follows: pFA6a-*ura4MX4* (2,387 bp), pFA6a-*arg3MX4* (2,540 bp), pFA6a-*his3MX4* (2,645 bp), and pFA6a-*leu1MX4* (2,185 bp). DNA was loaded onto a 0.8% Agarose (Bioline) gel (1× TAE) containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich Co.).