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Research Article

Three novel antibiotic marker cassettes for gene disruption and marker switching in *Schizosaccharomyces pombe*

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Abstract

The ease of construction of multiple mutant strains in *Schizosaccharomyces pombe* is limited by the number of available genetic markers. We describe here three new cassettes for PCR-mediated gene disruption that can be used in combination with commonly used fission yeast markers to make multiple gene deletions. The *natMX6, hphMX6* and *bleMX6* markers give rise to resistance towards the antibiotics nourseothricin (NAT), hygromycin B and phleomycin, respectively. The cassettes are composed of exogenous sequences to increase the frequency of integration at targeted loci, and have a structure similar to the commonly used *pFA6a–kanMX6* modular plasmid system. This allows a simple exchange of the *kanMX6* marker in existing strains with any of the three new cassettes. Alternatively, oligonucleotide primers designed for the modular *kanMX6* cassettes can be used to make the transforming PCR fragments for gene disruption. We illustrate the construction of a mutant strain with six independent gene disruptions, using the novel antibiotic cassettes in combination with existing genetic markers. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: ***Schizosaccharomyces pombe*; selectable markers; gene disruption; nourseothricin; hygromycin B; phleomycin**

Introduction

Selectable genetic markers are an important tool in the construction and analysis of targeted *Schizosaccharomyces pombe* mutants. Most commonly, selectable markers are used in the form of polymerase chain reaction (PCR)-amplified cassettes, which are integrated into the genome by means of flanking sequences homologous to the targeted locus (Rothstein, 1983; Grimm and Kohli, 1988; Bähler *et al*., 1998). Ideally, the marker cassettes allow for efficient selection without affecting any cellular functions, and display no sequence homology with the wild-type genome to reduce the frequency of integration at non-targeted loci.

Auxotrophic markers have been commonly used in *Sz. pombe*, including *ura4, his3, his7, arg6* and *Saccharomyces cerevisiae LEU2* complementing *leu1* (Grimm *et al*., 1988; Apolinario *et al*., 1993; Burke and Gould, 1994; Toh-e, 1995; Waddell and Jenkins, 1995). Phenotypic complementation by these markers requires that all strains have the corresponding auxotrophic genetic background. Further limitations of auxotrophic markers include unwanted phenotypic effects, such as growth defects and the presence of sequence homology between the marker and the genome in some cases.

An alternative to auxotrophic markers are antibiotic resistance markers, of which the *kanMX6* cassette is the only example widely used in *Sz. pombe*. The *kanMX6* cassette is composed of promoter and terminator sequences from *Ashbya gossypii*, and the *Escherichia coli* transposon Tn903 *kanR*

gene (Wach *et al*., 1994; Bähler *et al*., 1998; Longtine *et al*., 1998). The presence of *kanMX6* gives rise to strong, selectable resistance to the antibiotic geneticin (G418) but is not known to affect any other phenotypes. Since it consists entirely of exogenous sequences, the *kanMX6* cassette displays no sequence homology with the fission yeast genome, thereby reducing the frequency of integration at non-targeted loci.

In *Sz. pombe*, the number of available selectable markers is restricted, putting limitations on the construction of multiple mutants. We describe here three novel antibiotic resistance cassettes that can be used for the PCR-mediated construction of gene disruptions, viz. *natMX6, hphMX6* and *bleMX6*, which confer resistance to nourseothricin, hygromycin B and bleomycin, respectively. In combination with other commonly used selectable markers, the availability of three novel selectable markers enabled the relatively straightforward construction of a fission yeast strain with six independent gene disruptions. The novel markers share their genetic structure with the modular *kanMX6* cassette system described by Bähler *et al*. (1998), allowing for efficient marker switching in strains containing *kanMX6*. A particular advantage of nourseothricin selection is that it can be used efficiently in the presence of minimal media. Further applications of the *natMX6* nourseothricin resistance marker for strain construction are described in Van Driessche *et al*. (2005).

Materials and methods

Media and antibiotics

Media as previously described (Moreno *et al*., 1991) were supplemented with stock solutions of antibiotics to obtain final concentrations of 100 μg/ml unless stated otherwise. 1000× nourseothricin stock solutions were made by dissolving ClonNAT powder (Werner Bioagents) in distilled water, followed by filter-sterilization and storage at −20 °C. Aqueous solutions of hygromycin B and phleomycin were purchased from Cayla. For efficient selection, some batches of hygromycin B required higher concentrations (up to 400 μg/ml). Phleomycin plates were made fresh, as this antibiotic is relatively unstable. Phleomycin is genotoxic and should be used with care.

Construction of marker cassettes and *MX6* strains

The marker cassettes were constructed by PCR with the proof-reading polymerase Pwo (Roche). To make a *bleMX6* fragment, the P_{TEF} promoter was PCR-amplified using primers MX4/6cassUP (Table 1) and MX6UPrv (ggttgtttatgttcggatgtgatg); the T_{TEF} terminator using primers MX4/6cassDwn (Table 1) and MX6DWNfw (tcagtactgacaataaaaagattcttg). The *Streptoalloteichus hindustanus ble* open reading frame (ORF) was PCR-amplified from plasmid *pUT737* (Cayla), using primers zeoFWD (catcacatccgaacataaacaaccatggccaagttgaccagtg) and zeoRV (caagaatctttttattgtcagtactgatcagtcctgctcctcgg), creating 24 and 27 bp segments of sequence identity with the P_{TEF} and T_{TEF} fragments at either end of the *ble* ORF fragment. The *ble* ORF was then fused to the regulatory sequences by fusion PCR with the MX4/6cassUP and MX4/6cassDwn primers. PCR fragments for the construction of *natMX6* and *hphMX6* strains were amplified using the primers MX4/6cassUP and MX4/6cassDwn (Table 1) and the plasmids *pAG25* and *pAG32* (Goldstein and McCusker, 1999) as a template.

Table 1. PCR primers sequences for gene disruption and marker switching

Name	Purpose	Sequence
F2	Gene disruption	5′-(gene-specific sequence)-CGGATCCCCGGGTTAATTAA-3′
R1	Gene disruption	5′-(gene-specific sequence)-GAATTCGAGCTCGTTTAAAC-3′
MX4/6cassUP	Marker switching	5′-GACATGGAGGCCCAGAATAC-3′
MX4/6cassDwn	Marker switching	5′-TGGATGGCGGCGTTAGTATC-3′

Primers F2 and R1 are to be used with the *pFA6a–natMX6, pFA6a–hphMX6* or *pFA6a–bleMX6* plasmids for gene disruption and are identical to primers described in Bähler *et al.* (1998) and Van Driessche *et al.* (2005). DNA fragments produced by PCR amplification of the novel cassettes on the same plasmids using MX4/6cassUP MX4/6cassDwn primers can be used for marker switching in strains containing the *kanMX6* cassette, or for diagnostic PCR (see Figure 2b).

The PCR fragments representing the *natMX6, hphMX6* and *bleMX6* cassettes were then used for marker switching in strains containing the *kanMX6* marker, such as *rev3::kanMX6* (see Figure 1 for strains used). The DNA fragments were transformed into *kanMX6* strains using the lithium acetate method, as described in Bähler *et al.* (1998). Transformed cells were plated out on rich agar without antibiotics, incubated overnight at 30 °C, and replica-plated using velvet onto selective agar plates. Resistant colonies were obtained after 3 days incubation at 30 °C. Marker switching was verified by testing for geneticin sensitivity in the previously geneticin-resistant strains, and by diagnostic PCR.

Construction of *pFA6a–MX6* plasmids

For cloning the cassettes, genomic DNA was prepared from *natMX6-, hphMX6-* and *bleMX6*-containing yeast strains using Qiagen kits, and used as templates to PCR-amplify the *MX6* cassettes with MX4/6cassUP and MX4/6cassDwn primers (Table 1). The PCR fragments were gel-purified, digested with *Bgl*II–*Eco*RV and cloned into the backbone of *Bgl*II–*Eco*RV-digested, gel-purified *pFA6a–kanMX6*. The plasmid inserts were verified by sequencing. The *pFA6a–natMX6, pFA6a–hphMX6* and *pFA6a–bleMX6* plasmids were shown to be suitable for use as templates in PCR-mediated marker switching and gene disruption, using conditions similar to those described by Bähler *et al.* (1998). Efficient PCR amplification of *natMX6* and *bleMX6* requires the addition of 5% DMSO (Sigma) in the reaction mixture.

Additional information, including the sequences of the plasmids, is available at the collaborative website Biotwiki (**http://www.biotwiki.org/twiki/bin/view/Main/NewMarkers**). Plasmids will be made available through EUROSCARF together with *pFA6a* plasmids for gene tagging, described in Van Driessche *et al.* (2005) (**http://www.uni-frankfurt.de/fb15/mikro/euroscarf/**).

Results and discussion

Effect of antibiotics on *Sz. pombe*

In order to develop novel antibiotic resistance markers, we first assessed the effects of three antibiotics on fission yeast: nourseothricin (NAT), hygromycin B and phleomycin. Nourseothricin (NAT) is an inhibitor of ribosomal protein synthesis produced by *Streptomyces noursei* that induces miscoding during translation in a wide range of prokaryotic and eukaryotic organisms. NAT resistance can be conferred by the *S. noursei nat1* gene, encoding an N-acetyltransferase that monoacetylates nourseothricin (Haupt and Thrum, 1985; Krugel *et al.*, 1993) and has previously been used as a selectable marker in other yeast organisms (Goldstein and McCusker, 1999; McDade and Cox, 2001).

Hygromycin B is an aminoglycoside produced by *Streptomyces hygroscopicus* that inhibits translocation during translation (Cabanas *et al.*, 1978). Killing aerobic prokaryotes as well as eukaryotes, it is commonly used in *E. coli* and mammalian cell culture. Hygromycin B is stable in solution although sensitive to very acidic conditions. Hygromycin resistance is encoded by the 1 kb *hph* gene isolated from *Klebsiella pneumoniae* (Gritz and Davies, 1983).

Phleomycin is a complex of related copper-chelated glycopeptide antibiotics produced by *Streptomyces verticillus*. It binds DNA through intercalation and causes double-strand DNA breaks when activated by ferrous ions or oxygen. Phleomycin is stable in powder form but less so in solution, especially at low pH and high temperature. It acts on a broad range of organisms and has been used in bacteria, yeasts, plant and mammalian cells. Phleomycin resistance is conferred by the 375 bp *S. hindustanus ble* gene, which encodes a 14 kDa protein with strong affinity for antibiotics of the phleomycin family (Gatignol *et al.*, 1988; Drocourt *et al.*, 1990).

The growth inhibitory efficiency of these three antibiotics on *Sz. pombe* cells was assessed by spot tests (Figure 1a). At a concentration of 100 μg/ml in rich medium, the three antibiotics tested killed *Sz. pombe* cells more efficiently than geneticin. At 100 μg/ml in minimal plates, NAT had a strong growth inhibitory effect, in contrast to geneticin, hygromycin B and phleomycin, which were ineffective. After being kept at room temperature for 2 weeks, rich plates containing NAT or hygromycin B retained their capacity for inhibiting cell growth, but phleomycin plates had lost much of their activity (data not shown).

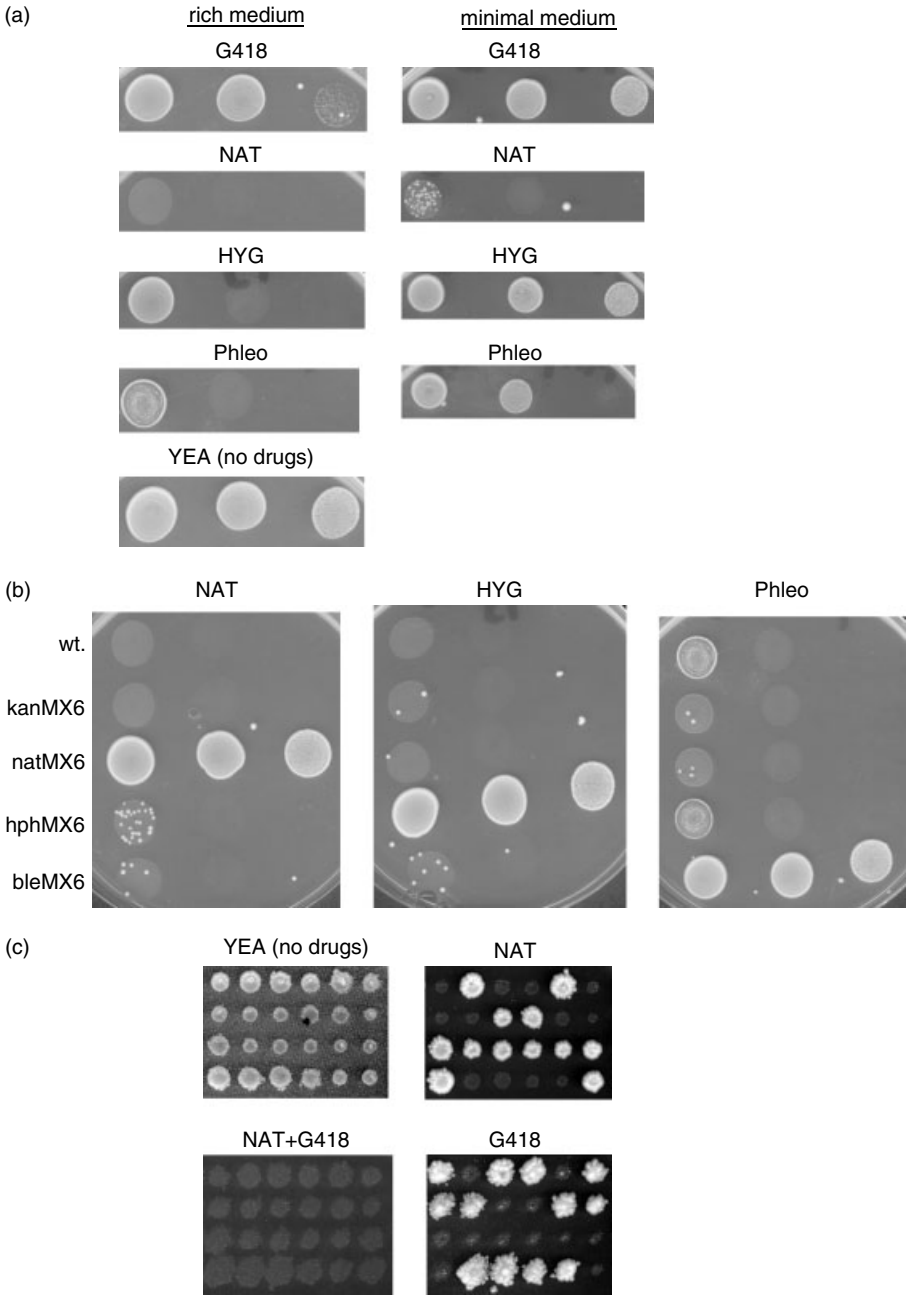

Figure 1. Sensitivity and resistance of *Sz. pombe* cells to nourseothricin, hygromycin B and phleomycin. (a) A 20-fold dilution series of wild-type *Sz. pombe* cells was spotted onto plates containing 100 μg/ml of either nourseothricin (NAT), hygromycin B (HYG), phleomycin (Phleo) or geneticin (G418), or no antibiotic. Plates were incubated at 30°C for 3 days. Cell density in the middle spot is similar that of a typical transformation experiment (10^7 cells/plate). At unusually high cell density, antibiotic-sensitive cells are sometimes able to grow. (b) A 20-fold dilution series of wild-type (wt), *hus1-MYC::kanMX6 (kanMX6), hus1-MYC::natMX6 (natMX6), rev3::hphMX6 (hphMX6) and dinB::bleMX6* (bleMX6) cells were spotted onto rich agar plates containing 100 μg/ml of either nourseothricin, hygromycin B or phleomycin. (c) Tetrad linkage analysis of *hus1-MYC::kanMX6 x hus1-MYC::natMX6*. Spores were dissected on non-selective YEA plates and the resulting colonies were replica-plated on YEA plates containing either nourseothricin or geneticin, or nourseothricin and geneticin

Construction of antibiotic-resistant strains

Sz. pombe strains resistant to the tested antibiotics were generated by integrating suitable resistance genes into the genome. DNA fragments consisting of a resistance gene ORF, flanked by P_{TEF} promoter and T_{TEF} terminator sequences from *A. gossypii*, were generated by PCR amplification to mimic the genetic architecture of the *kanMX6* cassette described by Bähler *et al*. (1998) (Figure 2c). Due to the 420 bp and 250 bp homology in the promoter and terminator regions shared by all *MX6* cassettes, transformation of these DNA fragments into strains which carried a gene disruption using the *kanMX6* cassette enabled marker switching through homologous recombination. Upon transformation of a *kanMX6*-marked strain with the novel cassettes, integrants were selected for in the presence of the corresponding antibiotic. Successful marker switching was tested for by assessing

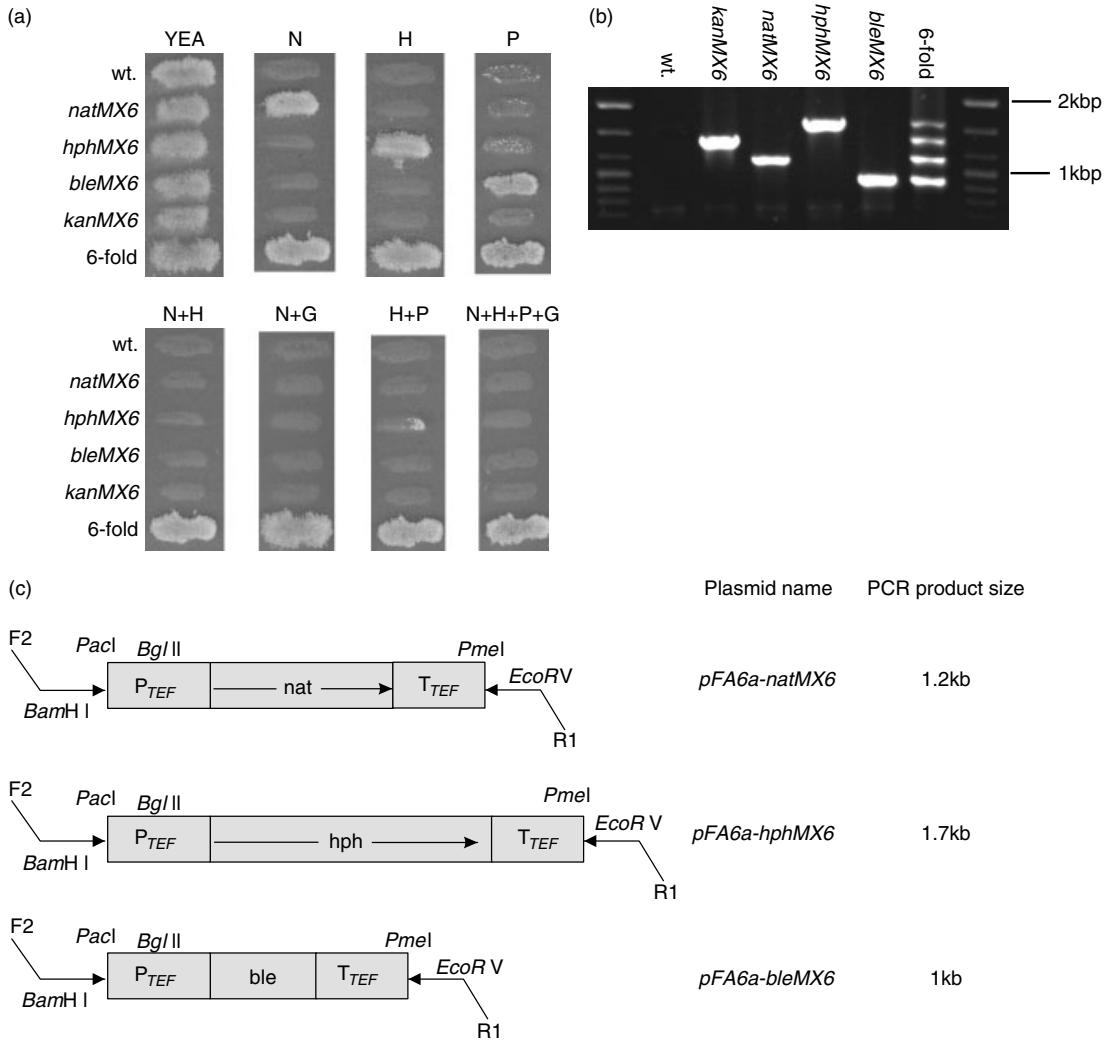

Figure 2. Six-fold mutant and schematic representation of the MX6 antibiotic marker cassettes. (a) Cells were patched on rich agar and replica-plated onto plates containing nourseothricin (N), hygromycin B (H), phleomycin (P) or geneticin (G) at a concentration of 100 μg/ml. The strains used were wild-type (*wt: leu1-32 ura4-D18 ade6-704 h*$^-$), the single mutants *hus1-MYC::kanMX6 (kanMX6), hus1-MYC::natMX6 (natMX6), rev3::hphMX6 (hphMX6), dinB::bleMX6 (bleMX6)* and the six-fold mutant strain *hus1-MYC::natMX6 rev3::hphMX6 dinB::bleMX6 eso1::kanMX rad13::ura4 uve1::LEU2*. (b) The presence of marker cassettes in the strains used in (a) was verified by PCR from genomic DNA, using the MX4/6cassUP and MX4/6cassDwn primers. (c) The structure of the *natMX6, hphMX6* and *bleMX6* modules

sensitivity to geneticin (i.e. loss of *kanMX6*), confirming integration over the *kanMX6* cassette in the *natMX6, hphMX6* and *bleMX6* strains.

The specificity of the resistance trait was assessed by spot tests (Figure 1b). *natMX6, hphMX6* and *bleMX6* marked strains were each specifically resistant to the corresponding drug. The *kanMX6* marker did not confer resistance to any other antibiotic. No unwanted growth inhibitory effects or other phenotypic defects were observed in cells containing any of the novel markers.

To verify that antibiotic resistance requires only a single copy of the resistance cassettes, the *natMX6, hphMX6* and *bleMX6* strains were crossed with the respective parental *kanMX* strains, and tetrad dissection was performed. Tetrad linkage analysis showed that nourseothricin and geneticin resistance displayed 2 : 2 segregation and double resistance was never observed (Figure 1c), suggesting that nourseothricin resistance was encoded at a single locus identical to the geneticin resistance locus. Equivalent results were obtained for hygromycin B and phleomycin resistance (not shown).

Construction of a mutant strain with six independent markers

Next we tested whether the three novel antibiotic markers could be used in combination with each other and with existing commonly used markers to construct multiple mutants. In a sequence of crosses, single mutants containing either one of the novel markers (*hus1-MYC::natMX6, rev3::hphMX6, dinB::bleMX6*), the geneticin resistance marker (*eso1::kanMX6*) or two commonly used auxotrophic markers (*rad13::ura4, uve1::LEU2*) were crossed, random spore analysis was performed and progeny with the highest number of genetic markers were isolated. Multiple mutants could be selected on plates containing several antibiotics (Figure 2a). A six-fold mutant, containing the four MX6 antibiotic markers in addition to *ura4* and *LEU2*, was isolated (*hus1-MYC::natMX6 rev3::hphMX6 dinB::bleMX6 eso1::kanMX rad13::ura4 uve1::LEU2*). Given the size difference between the four antibiotic resistance cassettes, their presence could be verified by PCR (Figure 2b).

Cloning of the marker cassettes into *pFA6a* plasmids for gene disruption

The three novel antibiotic resistance cassettes were then cloned into the *pFA6a* plasmids. The *natMX6, hphMX6* and *bleMX6* fragments were PCR-amplified from genomic DNA of the corresponding mutants using the MX4/6cassUP and MX4/6cassDwn primers (Table 1). PCR fragments were digested with *Bgl*II–*Eco*RV and cloned into the backbone of *pFA6a–kanMX6*. These plasmids have been used as a PCR template for the construction of mutants by targeted gene disruption with gene-specific long primers (F2 and R1; Table 1), as well as for marker switching in existing *kanMX6* strains using the non-gene-specific primer pair MX4/6cassUP and MX4/6cassDwn (Table 1).

Conclusion

We describe three novel antibiotic marker cassettes for *Sz. pombe: natMX6, hphMX6* and *bleMX6*. We show that these markers can be used in combination with existing markers to construct multiple mutant strains. A particular advantage of the *natMX6* –nourseothricin system is that it can be used in conditions where minimal medium is required, such as *ura4* and *LEU2* plasmids or the thiamine-regulatable *nmt* promoter. A *natMX6*-based thiamine regulatable module is described in Van Driessche *et al*. (2005).

The *natMX6, hphMX6* and *bleMX6* marker cassettes are available in *pFA6a* plasmids suitable for gene disruptions. These three plasmids are compatible with the modular *kanMX6* system (Bähler *et al*., 1998) and only differ from *pFA6a–kanMX6* in that the *kan* ORF is replaced by a *nat1, hph* or *ble* ORF. As a result, gene-specific long primers designed for the construction of *kanMX6* deletion strains can be used with the novel markers. More importantly, the novel antibiotic resistance cassettes can be amplified with the MX4/6cassUP and MX4/6cassDwn primers to generate fragments suitable for marker switching of any gene disruption or epitope tagged strain containing the *kanMX6* marker.

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