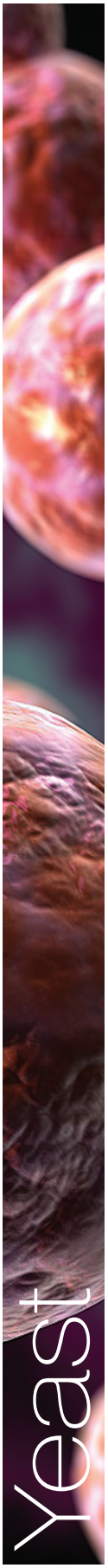

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Review

Saccharomyces cerevisiae Shuttle vectors

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Abstract

Yeast shuttle vectors are indispensable tools in yeast research. They enable cloning of defined DNA sequences in *Escherichia coli* and their direct transfer into *Saccharomyces cerevisiae* cells. There are three types of commonly used yeast shuttle vectors: centromeric plasmids, episomal plasmids and integrating plasmids. In this review, we discuss the different plasmid systems and their characteristic features. We focus on their segregational stability and copy number and indicate how to modify these properties.

Keywords: ***Saccharomyces cerevisiae*; plasmids; shuttle vectors; genomic integration; copy number; plasmid loss**

Introduction

Control of gene copy number is a prerequisite for quantitative yeast experimentation and is important for basic research, biotechnology and synthetic biology (Mileyko et al., 2008; Blount et al., 2012; Da Silva and Srikrishnan, 2012). In *Saccharomyces cerevisiae*, various plasmid systems can be used to introduce heterologous genes with a defined copy number.

Most plasmids used in yeast research are so-called 'shuttle vectors'. They can be constructed, manipulated and analysed in *Escherichia coli* as well as in *S. cerevisiae* (Ma et al., 1987; Oldenburg et al., 1997). To facilitate cloning, they usually contain a stretch of restriction enzyme recognition sites, called the polylinker or multiple cloning site (MCS). For their maintenance in *E. coli*, a suitable origin of replication (*ori*) and a selectable marker gene are required. High-copy *ori* sequences, such as ColE1, are utilized when high plasmid yields are desired (del Solar et al., 1998; Gietz and Sugino, 1988; Sikorski and Hieter, 1989). However, if the plasmid or its gene products exert a heavy burden to the host cell, low-copy *ori* sequences can be beneficial. For the selection of plasmid-carrying cells, antibiotic resistance genes like the β-lactamase gene (So et al., 1975) are commonly used.

In *S. cerevisiae*, shuttle vectors are maintained either extrachromosomally or by integration into the genome (see following three sections). For selection, the plasmids bear marker genes. Commonly used marker types are auxotrophic markers, autoselection systems and dominant markers (Table 1; for a comprehensive review see Siewers, 2014).

Auxotrophic marker genes code for a metabolic enzyme and complement a corresponding mutation in the host yeast strain. Therefore, only plasmid-carrying yeast cells can grow in media where the end product of the respective biosynthesis pathway is absent. Auxotrophic marker genes originate from *S. cerevisiae* or related species and contain either their original promoter and terminator sequences or heterologous ones (Hinnen et al., 1978; Weinstock and Strathern, 1993; Wach et al., 1994, 1997). As stated above, the use of

Table 1. Common selectable yeast marker genes

Marker gene	Mode of action	Medium condition	Counterselective agent	References
Auxotrophic marker genes				
URA3	Complements a non-functional *ura3* allele	w/o uracil	5-Fluoro-orotic acid	Bach *et al.* (1979), Struhl *et al.* (1979), Boeke *et al.* (1984)
LEU2	Complements a non-functional *leu2* allele	w/o leucine	—	Ratzkin and Carbon (1977), Hinnen *et al.* (1978)
HIS3	Complements a non-functional *his3* allele	w/o histidine	—	Struhl and Davis (1977)
TRP1	Complements a non-functional *trp1* allele	w/o tryptophan	5-Fluoroanthranilic acid	Struhl *et al.* (1979), Toyn *et al.* (2000)
ADE2	Complements a non-functional *ade2* allele	w/o adenine	—	Stotz and Linder (1990)
LYS2	Complements a non-functional *lys2* allele	w/o lysine	α-Aminoadipate	Eibel and Philippsen (1983), Simchen *et al.* (1984), Chattoo *et al.* (1979)
MET15	Complements a non-functional *met15* allele	w/o methionine	Methyl mercury	Brachmann *et al.* (1998), Singh and Sherman (1974)
Autoselection systems				
URA3	In a *fur1*Δ *ura1*Δ background, *URA3* is essential	Any medium	5-Fluoro-orotic acid	Napp and Da Silva (1993)
FBA1	Complements *fba1*Δ	Any medium	—	Compagno *et al.* (1993)
POT/TPI	Complements *tpi1*Δ	Fermentable carbon source	—	Kawasaki (1984), Kawasaki and Bell (1999)
CDCx	Complements a non-functional *cdc4*, *cdc9* or *cdc28* allele	Any medium	—	Unternahrer *et al.* (1991), Geymonat *et al.* (2007), Siewers (2014)
Dominant marker genes				
kan	Inactivates the translation inhibitor G418 by phosphorylation	200–350 μg/ml G418, w/o ammonium sulphate	—	Jimenez and Davies (1980), Wach *et al.* (1994), Taxis and Knop (2006)
hph	Inactivates the translation inhibitor hygromycin B by phosphorylation	100 μg/ml hygromycin B	—	Gritz and Davies (1983), Goldstein and McCusker (1999), Taxis and Knop (2006)
nat	Inactivates the translation inhibitor nourseothricin by acetylation	100 μg/ml nourseothricin	—	Goldstein and McCusker (1999), Taxis and Knop (2006)
pat	Inactivates the glutamine synthesis inhibitor bialaphos	200 μg/ml bialaphos in media lacking glutamate	—	Goldstein and McCusker (1999)
ble	Inactivates the DNA damage-inducing agent phleomycin	7.5 μg/ml phleomycin	—	Gueldener *et al.* (2002)
amdSYM	Allows growth on acetamide as sole nitrogen source	—	Fluoroacetamide	Solis-Escalante *et al.* (2013)
tk	Allows growth in the presence of antifolates	Antifolate mix, non-fermentable carbon source	5-Fluorodeoxyuridine	Alexander *et al.* (2014)

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auxotrophic marker genes requires a host strain with a corresponding genotype, but extensive collections of *S. cerevisiae* strains with multiple selectable auxotrophies are available (Thomas and Rothstein, 1989; Brachmann et al., 1998). Additionally, gene disruption techniques can be used to generate further auxotrophies in any laboratory strain or natural isolate (Baudin et al., 1993; Lorenz et al., 1995; Giaever et al., 2002).

Autoselection systems are based on marker genes which are essential in any (or almost any) nutritional environment; ideally, their absence cannot be compensated by media components and is inevitably lethal. Yeast strains with the respective genetic mutation can only survive if the gene is supplied by the plasmid. Although these systems are rarely used in basic research, they are attractive tools for large-scale production in biotechnology, as the choice of growth media is unrestricted and the use of antibiotics can be avoided. For example, insulin expression in *S. cerevisiae* was initially performed in strains bearing a mutation in the *TPI1* (triose phosphate isomerase) gene (Thim et al., 1986; Kjeldsen et al., 1996), which renders the cells inviable in the presence of glucose as the sole carbon source. The insulin expression plasmid complements the mutation, as it carries the corresponding gene from *Schizosaccharomyces pombe* (*POT1*), restoring the cells' ability to grow on glucose. Another commonly used system enables the usage of a *URA3* plasmid in any growth condition. This system requires triple mutant yeast strains blocking the pyrimidine biosynthesis (*ura3*) and salvage pathways (*fur1 urk1*) (Napp and Da Silva, 1993). In this setting, Ura3p is essential and can be complemented with a plasmid-borne *URA3* gene. Newer autoselection systems are based on complementation of essential cell cycle regulators. They also allow for the co-transformation of multiple plasmids (Geymonat et al., 2007).

Dominant marker genes enable yeast cells to grow in special chemical environments. Commonly, these genes code for enzymes that inactivate toxic compounds, such as antibiotics. Alternatively, they code for gene products enabling the cells to grow on uncommon nutrient sources (Solis-Escalante et al., 2013). The selection marker cassette consists of the coding sequence flanked by promoter and terminator sequences (Gritz and Davies, 1983; Hadfield et al., 1986; Wach et al., 1994; Goldstein and McCusker, 1999; Taxis and Knop, 2006). Heterologous promoters and terminators are preferable, as they preclude unwanted recombination with genomic sequences. The use of dominant markers does not require a special genotype of the host strain. This makes them especially useful when working with prototrophic strains, like natural isolates or some industrial strains.

Some marker genes allow counterselection (Table 1). Counterselection is the phenomenon when the marker gene converts a non-toxic substrate into a toxic product. Under appropriate conditions, the presence of such a substrate restricts growth to yeast cells that lost the function of the corresponding marker gene. In principle, every marker gene cassette can be made counterselectable by including either an inducible growth inhibitory sequence (Akada et al., 2002) or fusing the marker gene to thymidine kinase (Alexander et al., 2014). One application of counterselection is the recycling of auxotrophic markers during repetitive yeast transformations (see 'Other plasmid-based genome engineering tools', below).

Yeast centromeric plasmids

Features of yeast centromeric plasmids

Yeast centromeric plasmids (YCps) exploit the cell's endogenous replication and chromosome segregation machinery to persist in yeast cells like mini-chromosomes. To this end, YCps bear two characteristic sequences: autonomously replicating sequences (*ARS*) and centromeric (*CEN*) sequences (Figure 1a). *ARS* sequences are the genomic sites where DNA replication is initiated exactly once during the S-phase of every cell cycle (Stinchcomb et al., 1979; Fangman et al., 1983; Brewer and Fangman, 1987). *CEN* sequences are the attachment points for kinetochore complexes, which direct chromosome segregation along the mitotic spindle apparatus (Westermann et al., 2007).

Segregational stability and copy number of yeast centromeric plasmids

Both *ARS* and *CEN* sequences have to be present for stable plasmid maintenance and correct plasmid distribution during cell division (Murray and

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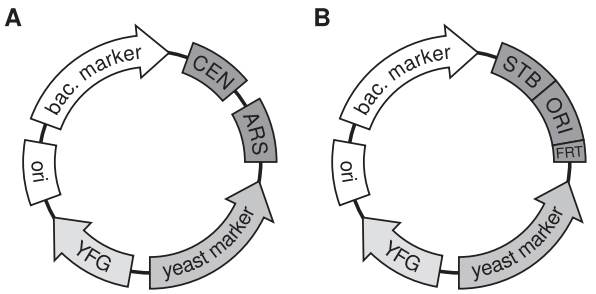

Figure 1. Extrachromosomal yeast plasmids. (a) Yeast centromeric plasmid (YCp). For maintenance in yeast, a selection marker, a centromeric (*CEN*) and an autonomously replicating sequence (*ARS*) are present. (b) Yeast episomal plasmid (YEp). For maintenance in yeast, 2μ plasmid-derived *STB* and *ORI* sequences are present. For maintenance in *E. coli*, both plasmid types contain a bacterial selection marker (bac. marker) and replication origin (ori). YFG, your favourite gene; FRT, Flp1p recombinase recognition site (see text for details)

Szostak, 1983; Clarke and Carbon, 1980; Hieter et al., 1985). YCps are maintained in the host cell with about one copy per haploid genome when averaged over the cell population (Clarke and Carbon, 1980; Tschumper and Carbon, 1983). However, the copy number in individual cells varies: a substantial fraction of cells do not carry any plasmids and many cells carry more than one (Ryan et al., 2014; Lee et al., 2015; Gnügge et al., 2016).

The presence of the *CEN* sequence confers a high segregational stability during mitosis (Murray and Szostak, 1983; Hieter et al., 1985). However, asymmetric plasmid segregation can occur with frequencies of about 10% per plasmid pair (Gnügge et al., 2016). Another – less frequent – mechanism that leads to copy number changes is the failure of plasmid replication (Murray and Szostak, 1983; Hieter et al., 1985). The combination of these two mechanisms results in the observed copy number heterogeneity and leads to the generation of plasmid-free cells in about 3–5% per cell division. Interestingly, there is a correlation between plasmid size and segregational stability. The larger the YCp, the less frequent is its unequal distribution during cell division (Hieter et al., 1985).

Transcription through the *CEN* sequence renders it non-functional, as kinetochore assembly is physically blocked (Chlebowicz-Sledziewska and Sledziewski, 1985; Hill and Bloom, 1987). This drastically increases the asymmetric plasmid segregation rate and leads to the accumulation of cells with high copy numbers and of cells lacking any plasmid (Murray and Szostak, 1983). If multiple different YCps are present in the same cell they reduce each others' segregational stability. Furthermore, they can impair the host cell's fitness (Futcher and Carbon, 1986; Runge et al., 1991).

Yeast episomal plasmids

The endogenous yeast 2μ plasmid

Yeast episomal plasmids (YEps) are based on sequences from a natural yeast plasmid. This plasmid is present in most wild-type and laboratory *S. cerevisiae* strains and has a length of 6318 bp (Hartley and Donelson, 1980). Alluding to its contour size, it was termed 2μm or 2μ plasmid (Stevens and Moustacchi, 1971; Guerineau et al., 1971). The plasmid is cryptic, as it is not associated with any apparent phenotype and confers no selective advantage to its host cell; nevertheless, its loss is very rare (Futcher and Cox, 1983). The 2μ plasmid persists in yeast cells with 40–80 copies per haploid genome (Clark-Walker and Miklos, 1974; Gerbaud and Guerineau, 1980; Futcher and Cox, 1984). The plasmid copies are not homogeneously distributed in the nucleus, but are found in a few clusters (Scott-Drew and Murray, 1998; Velmurugan et al., 2000).

Replication and partitioning depend on special plasmid sequences and plasmid-encoded proteins (Figure 2). A replication origin (*ORI*) directs replication initiation exactly once in the S-phase of every cell cycle (Broach and Hicks, 1980; Zakian et al., 1979; Huberman et al., 1987; Brewer and Fangman, 1987). The detailed mechanism of plasmid partitioning is not yet fully understood (Chan et al., 2013), but it involves a *cis*-acting sequence (*STB*), plasmid-encoded DNA-binding proteins (Rep1p and Rep2p) and host cell factors (Broach and Hicks, 1980; Kikuchi, 1983; Jayaram et al., 1983; Murray and Cesareni, 1986). During cell division, a centromere-like chromatin structure is assembled at the *STB* locus and plasmid clusters are distributed between mother and daughter cells like chromosomes (Hajra et al., 2006; Ma et al., 2013).

In the case of unequal partitioning of 2μ plasmid clusters during cell division, a recombination-based plasmid amplification system can restore high copy numbers. The 2μ plasmid codes for a

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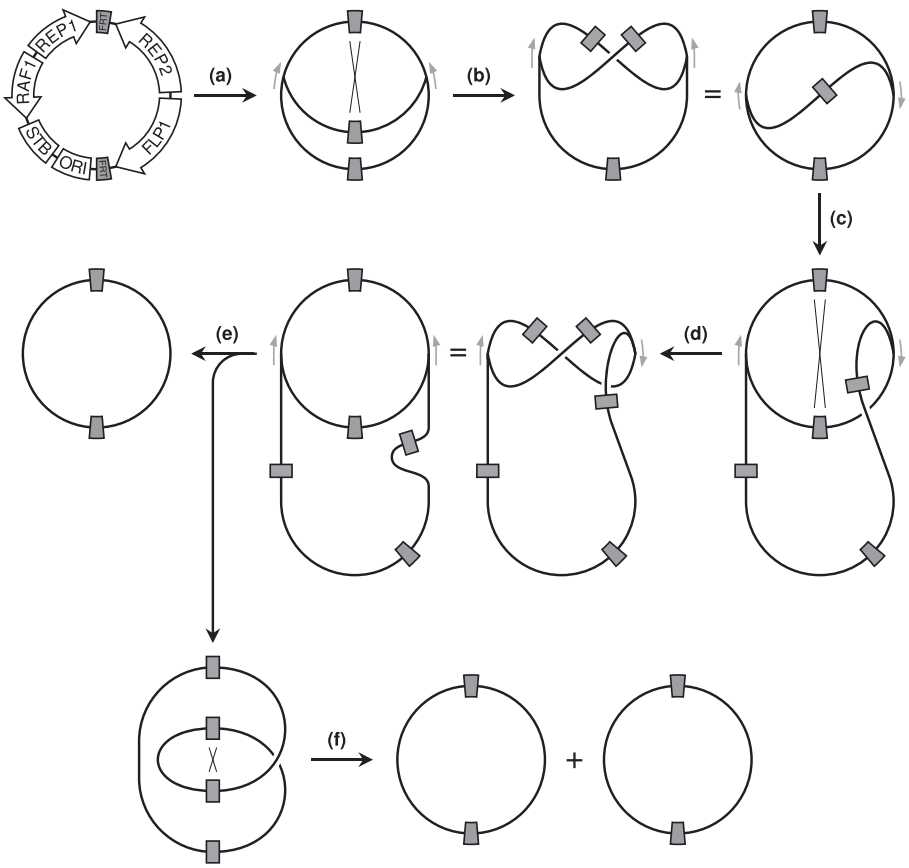

Figure 2. Recombination-based amplification of the 2μ plasmid. (a) The replication of the 2μ plasmid is initiated at the *ORI* sequence, which is located near one of the two *FRT* sites (grey boxes). (b) Flp1p mediates recombination between the *ORI*-distal *FRT* site and the already replicated *FRT* site. (c) The two replication forks (grey arrows) travel in the same direction around the 2μ plasmid, resulting in rolling-circle replication. In this step, more than the shown two concatenated copies can be generated. (d) Flp1p mediates a second recombination event between the *FRT* sites of the original 2μ plasmid. This restores converging replication fork travel directions. (e) Replication is terminated and a single 2μ plasmid as well as concatenated 2μ plasmids are released. (f) The latter are resolved by Flp1p-mediated recombination

site-specific recombinase (*FLP1*) and bears two corresponding recognition sites (*FRT* sites) (Broach and Hicks, 1980). The *FRT* sites are located asymmetrically relative to the *ORI* (Figure 2). Recombination during plasmid replication between the *FRT* site distal to the *ORI* and the already replicated *FRT* site proximal to the *ORI* results in unidirectional rolling circle replication and thereby plasmid amplification (Figure 2). A second recombination event can reset the replication fork directions, leading to bidirectional replication and amplification termination (Futcher, 1986; Volkert and Broach, 1986).

Whether such an amplification-inducing recombination event takes place depends on the expression level of the Flp1p recombinase. The *FLP1* expression level is regulated by Rep1p and Rep2p. The expression of *REP1* and *REP2*, in turn, depends on the plasmid copy number and is additionally regulated by a feedback system involving another plasmid-encoded protein (Raf1p) (Murray et al., 1987). Together, these proteins establish a plasmid copy number-sensing system that triggers plasmid amplification if the plasmid copy number drops (Volkert and Broach, 1986; Murray et al., 1987; Som et al., 1988).

Features of yeast episomal plasmids

There are two major classes of YEps that differ in the amount of 2μ sequences they contain (Broach, 1983; Rose and Broach, 1990). The first class of

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YEps is generated by the insertion of bacterial plasmid sequences and appropriate selection markers into the complete endogenous 2μ plasmid (Beggs, 1978). Disadvantages of these YEps are their bulky size, the reduced availability of unique restriction enzyme recognition sites and their frequent recombination with the endogenous 2μ plasmid, which can remove the inserted sequences. The so-called 'disintegration vectors' (pSAC plasmids) are a special class of these YEps (Chinery and Hinchliffe, 1989). They consist of the complete endogenous 2μ plasmid with inserted yeast marker, expression cassette and bacterial sequences (*ori* and selection marker). The bacterial sequences are flanked by inverted *FRT* sites and replace one of the 2μ-inherent *FRT* sites. Upon propagation in yeast cells, the bacterial sequences are excised by Flp1p-mediated recombination. These vectors are useful in the context of biotechnological production processes when the amount of heterologous sequences in the host organism needs to be minimized.

In the second class of YEps the only sequences present from the 2μ plasmid are the *ORI* and *STB* sequences (Hicks et al., 1979; Christianson et al., 1992) (Figure 1b). Therefore, such YEps have a smaller size and usually contain more unique restriction sites. The 2μ fragment used to construct these YEps often contains a *FRT* site allowing recombination with endogenous 2μ plasmids (Christianson et al., 1992). However, the *FRT* sequence can be mutated to render it non-functional (Andrews et al., 1986). Such a mutation has no impact on plasmid maintenance or copy number (Hill et al., 1986; Gietz and Sugino, 1988).

Segregational stability and copy number of yeast episomal plasmids

YEps need functional *STB* and *ORI* sequences for their stable maintenance. Transcription through *STB* and *ORI* sequences must be avoided, as this impairs their functionality (Murray and Cesareni, 1986; Bijovet et al., 1991). Furthermore, the *REP1* and *REP2* gene products are essential for YEp persistence (Broach and Hicks, 1980; Jayaram et al., 1983; Kikuchi, 1983). YEps containing the complete 2-μ sequence bear all necessary sequences in *cis*. Such YEps are self-sufficient, as long as the heterologous sequences are placed such that they do not interfere with the functionality of the plasmid elements (Chinery and Hinchliffe, 1989; Bijovet et al., 1991). They can be stably maintained in yeast strains lacking the endogenous 2μ plasmid (so-called cir^0 strains) (Futcher and Cox, 1984). YEps that contain only the *ORI* and *STB* sequences depend on the expression of *REP1* and *REP2* in *trans*. This is the case in host strains also containing the endogenous 2μ plasmid (so-called cir^+ strains). In cir^0 strains, *REP1* and *REP2* need to be constitutively expressed from other plasmids or genomic loci (Som *et al.,* 1988; Dobson et al., 1988).

While the endogenous 2μ plasmid is lost in less than 0.1‰ of the cell divisions (Futcher and Cox, 1983), YEps have a higher plasmid loss frequency. YEps that are created by the insertion of heterologous sequences into the complete 2μ plasmid are typically lost in about 1% of the cell divisions. YEps containing only the 2μ *ORI* and *STB* sequences have plasmid loss frequencies of 1–5% in cir^+ cells and of up to 50% in cir^0 cells if *REP1* and *REP2* expression in *trans* is absent (Christianson et al*.,* 1992; Futcher and Cox, 1984). The increased plasmid loss frequency of YEps can be attributed to decreased replication or amplification propensities and burdening overexpression of marker genes and other heterologous genes (Rose and Broach, 1990; Cakar et al., 1999; Karim et al., 2013).

YEps containing the complete 2μ sequence achieve 40–80 copies per cell, which is comparable to the endogenous 2μ plasmid copy number (Futcher and Cox, 1984). YEps containing only the *ORI* and *STB* sequences usually exist at 10–40 copies per cell (Futcher and Cox, 1984; Christianson et al., 1992; Karim et al., 2013; Gnügge et al., 2016). The employed yeast marker has a significant effect on the copy number, as it can represent a burden to the host cell, impede its growth and favour the accumulation of cells with lower plasmid numbers (Karim et al., 2013). A gene of interest cloned into a YEp can reduce the copy number in a similar way, especially when expressed from a strong promoter (Fang et al., 2011; Karim et al., 2013; Gnügge et al., 2016).

For some biotechnological production processes, high plasmid copy numbers are needed. In this context, YEp systems that reach several hundreds of copies per cell have been developed. When dealing with YEps containing the complete 2μ sequence, one approach is the stimulation of

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the physiological plasmid amplification system. This can be accomplished by direct overexpression of the *FLP1* gene or of the *RAF1* gene, which in turn releases Rep1p/Rep2p-mediated repression of the *FLP1* gene (Murray et al., 1987; Som et al., 1988).

Another strategy to increase the copy number is to exploit poorly expressed marker genes, which results in the selection of cells carrying many copies of the plasmid. Low marker expression can be achieved by low transcription or translation propensities of the marker gene or low stability of the marker gene product (Kawasaki and Bell, 1999; Chen et al., 2012). Most common is the use of auxotrophic marker genes with truncated promoters, such as *leu2d* or *ura3d* (Erhart and Hollenberg, 1983; Loison et al., 1989; Lopes et al., 1991). In the previously mentioned 'POT' plasmid example, the triose phosphate isomerase gene (*POT*) from *S. pombe* is weakly transcribed in *S. cerevisiae*, leading to a selection for cells with high plasmid load on media containing glucose as the sole carbon source (Kawasaki, 1984; Russell, 1985; Kawasaki and Bell, 1999). For poorly expressed dominant marker genes, the plasmid copy number can be tuned by titrating the drug dose (Lian et al., 2016).

Yeast integrative plasmids

Features of yeast integrative plasmids

Yeast integrative plasmids (YIps) are inserted into the host cell genome. Once integrated, YIps are replicated and transmitted to successor cells as part of a chromosome. Characteristic features of YIps are targeting sequences that are homologous to genomic loci. Depending on the yeast strain and the integration site, as little as 30 bp of homologous targeting sequence can be sufficient for the generation of correct transformants. However, targeting sequences usually consist of a few hundreds of base pairs to achieve reliable and efficient integrations (Manivasakam et al., 1995).

Integration mechanisms

YIps integrate into the host cell genome via homologous recombination (Symington et al., 2014; Kowalczykowski, 2015). Depending on the number and localization of the targeting sequences, the integration occurs via a single-crossover or a double-crossover recombination mechanism (Rothstein, 1991).

Most commonly used YIps exploit the single-crossover mechanism (Figure 3a). Such YIps contain a single continuous targeting sequence. In the resulting transformants the target site is duplicated. Prior to transformation the YIp is linearized within the targeting sequence using appropriate restriction enzymes. This increases the transformation efficiency and defines the genomic integration site (Orr-Weaver et al., 1981, 1983). If the YIp bears multiple sequences that are homologous to yeast genomic loci the sequences nearest to the cut define the preferred integration site (Hicks et al., 1979; Orr-Weaver et al., 1981). Targeting sequences do not need to be at the very ends of the linearized plasmid to direct efficient integration. If necessary, non-homologous sequences at the ends will be trimmed by recombination factors (Ma et al., 1987; Svetec et al., 2007).

YIps that integrate via a double-crossover mechanism are inserted into the host cell genome in a gene replacement fashion (Rothstein, 1983). They contain two targeting sequences flanking the part of the YIp that is to be integrated (Figure 3b) (Lee and Da Silva, 1997; Voth et al., 2001; Taxis and Knop, 2006; Gnügge et al., 2016; Wosika et al., 2016). Cutting outside the targeting sequences liberates the integrative part of the plasmid. Thereby, unnecessary plasmid sequences can be excluded from the integration.

Segregational stability and copy number of integrating yeast plasmids

The integration of a YIp via a double-crossover mechanism results in a genomic configuration without direct repeat sequences. Therefore, such YIps integrate with a single plasmid copy and are segregationally stable (Taxis and Knop, 2006).

The integration of a YIp via a single-crossover mechanism leads to a genomic configuration with the integrated YIp flanked by direct repeats of the target site (Figure 3a). This configuration has decreased structural stability as recombination between direct repeats is possible (Rothstein, 1991). Such YIps can be lost with frequencies of up to 1% per cell division (Hinnen et al., 1978; Hicks et al., 1979). Selection for the plasmid marker does

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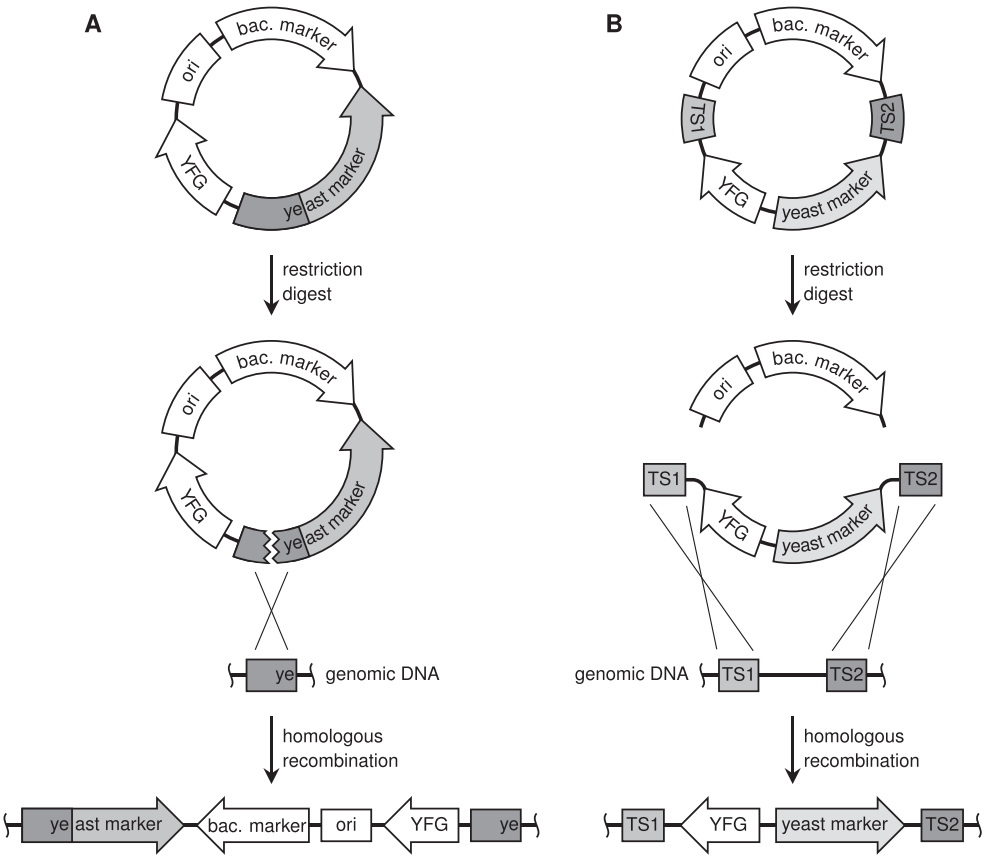

Figure 3. Yeast integrative plasmids (YIps). (a) YIps that integrate via a single-crossover recombination mechanism carry a single continuous targeting sequence, which is often a part of the marker gene in case of YIps with auxotrophic markers. The YIp is linearized within the targeting sequence by restriction digest. After integration the target site is duplicated. (b) YIps that integrate via a double-crossover mechanism contain two targeting sequences flanking the part of the vector that is to be integrated. Cutting outside the targeting sequences liberates the integrative part of the YIp. bac. marker, bacterial marker; ori, bacterial origin of replication; YFG, your favourite gene; TS, targeting sequence

not guarantee selection of intact transformants, since a part of the YIp can be looped out, while the selection marker is retained at the integration site. Moreover, single-crossover integrating plasmids often give rise to transformants carrying multiple tandem integrations (Orr-Weaver and Szostak, 1983; Gnügge et al., 2016). The prevailing copy number can be tuned by varying the concentration of the transforming plasmid (Plessis and Dujon, 1993).

Higher copy numbers can be achieved by directing the integration to sites that are abundant in the yeast genome, such as ribosomal DNA (rDNA) repeats (Szostak and Wu, 1979; Lopes et al., 1989; Fujii et al., 1990; Leite et al., 2013) and δ elements (Sakai *et al.,* 1990; Lee and Da Silva, 1997). Integration into abundant genomic target sites also increases the transformation efficiency, which supports the construction of industrial yeast strains that are often hard to transform (Szostak and Wu, 1979; Wilson et al., 1994).

The rDNA locus consists of 100–200 direct repeats of a 9.1 kb sequence that codes for the ribosomal RNAs and contains several transcribed and untranscribed spacer regions (Woolford and Baserga, 2013). Care has to be taken not to use the non-transcribed spacer region *NTS2* as the targeting sequence, since it contains an *ARS* sequence (Skryabin et al., 1984). Often, YIps carrying *NTS2* do not integrate into the yeast genome, but transform yeast cells as segregationally unstable extrachromosomal plasmids (Szostak and Wu, 1979).

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The δ elements are the long terminal repeat sequences of *Ty1* yeast transposons. They occur with more than 300 copies dispersed throughout the yeast genome (Kim et al., 1998). Multiple integrations usually occur into δ elements on different chromosomes. The crossing of transformants allows for the generation of cells with high copy numbers of integrated YIps (Sakai et al., 1991).

The copy number of YIps targeted to abundant genomic loci can be further increased if poorly expressed marker genes are used (see 'Yeast episomal plasmids', above) (Lopes et al., 1989, 1991; Maury et al., 2016). Furthermore, marker recycling (see 'Other plasmid-based genome engineering tools', below) can be exploited (Fujii et al., 1990). Transformants with several dozen integrated YIps are routinely created using these strategies independently of the respective insert (Yamada et al., 2010). As the integration is random, a set of different inserts in the same plasmid backbone can be integrated at once, allowing the probing of combinatorial libraries (Kato et al., 2013). For YIps carrying a dominant marker gene the copy number can be adjusted with the concentration of the selective compound used during the transformation process (Wang et al., 1996; Parekh et al., 1996; Parekh and Wittrup, 1997; Shusta et al., 1998).

Integration via a single-crossover mechanism usually leads to clusters of multiple direct tandem repeats of integrated plasmids (Lopes et al., 1991), but these configurations are structurally unstable. During prolonged cultivation, recombination events can reduce the initial plasmid copy number of transformants (Wang et al., 1996).

Series of shuttle vectors

Several series of YIps, YCps and YEps with different yeast selection markers as well as the corresponding yeast strains are available (Table 2).

The pRS series is the most commonly used shuttle vector series (Sikorski and Hieter, 1989; Christianson et al., 1992). It is based on the plasmid backbones of pBluescribe and pBluescript (Short et al., 1988; Alting-Mees and Short, 1989). The pRS plasmids are relatively small and have many unique in-frame restriction sites in their polylinker. They allow blue–white screening (α-complementation) (Ullmann et al., 1967; Vieira and Messing, 1982) for an easy identification of insert-bearing plasmids and have a bacterial *ColE1 ori* for high copy number propagation in *E. coli*. pRS vectors have a consistent design, which allows shuffling of DNA sequences between the polylinkers of different members of the series. The original pRS series contains the auxotrophic yeast marker genes *HIS3*, *TRP1*, *LEU2* and *URA3* (Sikorski and Hieter, 1989), but has been expanded with further auxotrophic and dominant marker genes (Sikorski and Boeke, 1991; Brachmann et al., 1998; Eriksson et al., 2004; Taxis and Knop, 2006; Chee and Haase, 2012). Furthermore, pRS derivatives with different bacterial selection markers have been constructed (Frazer and O'Keefe, 2007).

The pRG series of shuttle vectors consists of single- and multi-copy YIps, YCps and YEps (Gnügge et al., 2016). To achieve stable single-copy integrations, the YIps integrate via a double-crossover recombination mechanism. Multi-copy integrations are generated by targeting the plasmids to δ sites. All pRG plasmids consist of modular parts that are flanked by unique restriction sites. This not only allows for shuffling cloned DNA sequences between the polylinkers of different plasmids, but also an easy exchange or modification of every vector part. For example, YIps, YCps and YEps can be easily interconverted. Furthermore, vectors with new parts, such as yeast or bacterial markers, can be added to the series.

Various specialized shuttle vector series have been established (Da Silva and Srikrishnan, 2012). For example, series with pre-implemented expression cassettes were developed and used for biotechnological applications and complementation studies. In such vectors the polylinker is flanked by a constitutive or inducible promoter and a transcriptional terminator. Multiple unique restriction enzyme recognition sites in the polylinker facilitate the insertion of a coding sequence of interest (Mumberg et al., 1994, 1995; Fang et al., 2011; Shen et al., 2012). Additionally, vector series are available for the expression of multiple genes from one plasmid (Vickers et al., 2013). Other series of shuttle vectors allow recycling of the yeast marker (see next section)

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Table 2. Shuttle vector series

Series name	Plasmid types	Integration mechanism	Yeast markers	Comments	References
YCplac, YEplac, YIplac	YCp, YEp, YIp	Single-crossover	*URA3*, *TRP1*, *LEU2*	'RE-cleaned' yeast markers	Gietz and Sugino (1988)
YCp4xx, YEp4xx	YCp, YEp	—	*URA3*, *TRP1*, *LEU2*, *LYS2*, *HIS3*	Plasmids assembled by gap repair	Ma *et al.* (1987)
pRS	YCp, YEp, YIp	Single-crossover (double-crossover for dominant markers)	*URA3*, *TRP1*, *LEU2*, *LYS2*, *HIS3*, *ADE2*, *MET15*, *HIS2*, *ADE1*, *kan*, *hph*, *nat*, *pat*, *ble*	Classical and most popular series	Sikorski and Hieter (1989), Christianson *et al.* (1992), Brachmann *et al.* (1998), Taxis and Knop (2006), Chee and Haase (2012), Eriksson *et al.* (2004)
p4xxprom	YCp, YEp	—	*URA3*, *TRP1*, *LEU2*, *HIS3*	MCS is flanked by constitutive or inducible promoters and *CYC1* terminator	Mumberg *et al.* (1994, 1995)
pAG	YCp, YEp, YIp	Single-crossover	*URA3*, *TRP1*, *LEU2*, *HIS3*	Gateway™-compatible, MCS is flanked by *GAL1* *GPD* promoter and fluorescence or epitope tags	Alberti *et al.* (2007)
pXP	YCp, YEP	—	*URA3*, *TRP1*, *LEU2*, *leu2d*, *HIS3*, *MET15*	MCS is flanked by a constitutive or inducible promoter and the *CYC1* terminator. Expression cassette and marker can by amplified by PCR for integration and marker can be recycled using the Cre/*loxP* system	Fang *et al.* (2011), Shen *et al.* (2012)
pMG	YCp, YEp, YIp	Single-crossover	*URA3*, *TRP1*, *LEU2*, *HIS3*	MultiSite Gateway™-compatible	Nagels Durand *et al.* (2012)
EasyClone	YEp, YIp	Double-crossover	*URA3*, *LEU2*, *HIS3*, *LYS5*, *kan*	Compatible with uracil excision reaction-based (USER) cloning (Nour-Eldin *et al.,* 2006), Cre/*loxP*-mediated marker recycling	Jensen *et al.* (2014)
Yeast Toolkit	YCp, YEp, YIp	Double-crossover	*URA3*, *LEU2*, *HIS3*, *kan*, *nat*, *hph*, *ble*	Collection of parts to assemble genes and plasmids based on the modular cloning (MoClo) system (Weber *et al.,* 2011)	Lee *et al.* (2015)
D-POP	YIp	Double-crossover	*URA3*, *HIS3*, *LYS5*, *kan*, *nat*	Customized repeat sequences for scar-less marker recycling	Siddiqui *et al.* (2014)
pRG	YCp, YEp, YIp	Double-crossover	*URA3*, *LEU2*, *HIS3*, *MET15*, *LYS2*	'RE-cleaned' yeast markers, modular design, multi-copy integration into Ty1 δ sites	Gnügge *et al.* (2016)

(Jensen et al., 2014; Fang et al., 2011; Shen et al., 2012; Siddiqui et al., 2014), or are compatible with cloning standards, such as Gateway™, USER and MoClo (Alberti et al., 2007; Nagels Durand et al., 2012; Jensen et al., 2014; Lee et al., 2015).

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Other plasmid-based genome engineering tools

Marker recycling using repeat sequences

The number of successive transformations of a yeast strain with different YIps is limited by the number of selectable marker genes. A strategy to overcome this limitation is to eliminate the selection marker, once the YIp has been correctly integrated, allowing for repetitive reuse of the marker. This process is termed 'marker recycling' and relies on a recombination based loop-out of a counterselectable marker gene (Table 1). A spontaneous excision is possible when the marker gene is flanked by direct repeats. Classically, the construct contains the *URA3* marker gene flanked by 1.1 kb direct repeats originating from the *Salmonella typhimurium hisG* operon (Alani et al., 1987). As the repeats do not share any homology with yeast genomic sequences, mistargeted integrations are avoided and the marker-excising recombination is restricted to the marker-flanking repeats. Using 5-fluoro-orotic acid (5-FOA) for counterselection, cells that have lost the *URA3* marker gene are readily isolated (Boeke et al., 1984). A problem with this marker recycling approach is the accumulation of *hisG* sequences in the genome, which can lead to mistargeted integrations and genomic instability (Davidson and Schiestl, 2000). To overcome these shortcomings, customized repeat sequences can be used. Using customized repeat sequences and multiple counterselectable markers allows for the engineering of several genomic sites in parallel (Siddiqui et al., 2014).

Marker recycling using customized repeats has also been employed for polymerase chain reaction (PCR)-mediated genome alterations. By choosing the genomic sequence proximal to the integration site as the repeat sequence, scarless modifications were obtained (Akada et al., 2006; Kaneko et al., 2009; Solis-Escalante et al., 2013). Similarly, scarless genome alterations were achieved by providing a bridging oligo as a repair template. This so-called 'delitto perfetto' method requires a second transformation with oligonucleotides that are homologous to the sequences flanking the integrated counterselectable marker (Storici et al., 2001).

Marker recycling is more efficient when a DNA double-strand break (DSB) is created next to the repeat sequences, allowing even the removal of non-counterselectable markers (Storici et al., 2003; Khmelinskii et al., 2011; Solis-Escalante et al., 2014). Commonly, the endonucleases HO or I-SceI and their corresponding recognition site are used (Nickoloff et al., 1986; Fairhead and Dujon, 1993). In the case of the 'delitto perfetto' method, the marker removal can be enhanced by I-SceI cutting in the marker gene and tethering of the recombinogenic oligonucleotides to the nuclease via an aptamer structure (Ruff et al., 2014). Nuclease-stimulated and scarless marker removal could also be applied to the marker recycling of YIps. An example of such a marker cassette is the MX4blaster, where a marker, the inducible I-SceI nuclease, a recognition site and an inducible counterselection is integrated (Carvalho et al., 2013). The cassette is recycled by transformation with a DNA sequence recombining on each side of the cassette and induction of the counterselectable gene.

An elegant nuclease-based method that allows for integration of multiple sequences with a limited number of selectable markers is the so-called 'reiterative recombination' (Wingler and Cornish, 2011; Ostrov et al., 2013). The method is based on two YIps that carry a selection marker, inducible HO or I-SceI and the I-SceI or HO recognition site, respectively. Beginning from the second transformation, endonuclease expression is induced and mediates the integration of the next YIp into a part of the previously integrated YIp. The integration is accompanied by the substitution of the selection marker. Cycles of transformation, endonuclease induction and selection allow for the elongation of the stretch of integrated sequences.

Marker recycling using recombinases

Besides flanking repeats and recombinogenic oligonucleotides, site-specific recombinases can also be used for marker recycling. Commonly, the Cre/*loxP* recombination system from the bacteriophage P1 is employed for this purpose (Sauer, 1987; Johansson and Hahn-Haegerdal, 2002). The marker gene is flanked by direct repeats of the 34 bp *loxP* sequence. Recombination between the *loxP* sites can then be initialized using the Cre recombinase controlled by an inducible promoter to remove the marker gene. The inducible Cre gene is either supplied by another plasmid or

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included in the marker cassette (Agaphonov and Alexandrov, 2014). Vector series exist for multi-insert and multi-copy integrations and for the recycling of multiple markers at once (Xie et al., 2014; Maury et al., 2016; Jensen et al., 2014). Another marker recycling system is based on the yeast endogenous 2μ Flp1p recombinase (Storici et al., 1999). The marker gene is flanked by *FRT* sites and the *FLP1* recombinase gene is supplied by the endogenous 2μ plasmid (in cir^+ strains) or can be included in the marker cassette (in cir^0 strains).

All these enzyme-mediated site-specific recombination events leave scar sequences at the site of marker removal. As for the flanking directed repeat approaches, repetitive marker recycling leads to the accumulation of scars and can give rise to mistargeted integrations and genome instability (Davidson and Schiestl, 2000; Delneri et al., 2000). To avoid this, mutated *loxP* or *FRT* sequences can be used (Carter and Delneri, 2010; Storici et al., 1999).

CRISPR/Cas

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas (CRISPR-associated) system (Jiang and Marraffini, 2015) enables genomic modifications independent of selection markers (Jessop-Fabre et al., 2016). Cas9 is a programmable endonuclease, whose target site can be defined almost at will. The target site is determined by expressing a so-called guide RNA (gRNA) together with Cas9. The gRNA binds to Cas9 and directs its nucleolytic activity to its complementary DNA sequence (Jinek et al., 2012). The only limitation of the target site choice is the presence of a defined short sequence directly adjacent to the target sequence, the so-called protospacer adjacent motif (PAM), which is usually a trinucleotide sequence (Mojica et al., 2009).

Cas9 generates a double-stranded break (DSB) at the target site, which is a genotoxic lesion and reduces cell growth and survival (Bennett et al., 1993). Only cells that successfully repair the DSB regain normal growth. A DSB can be repaired by the error-prone non-homologous end joining (NHEJ) pathway, which can result in small insertions or deletions at the cut site (Symington et al., 2014). Alternatively, the DSB can be repaired by homologous recombination when donor DNA is supplied that contains homology up- and downstream to the cut site (DiCarlo et al., 2013; Ryan et al., 2014). If the DSB is repaired such that the target site or PAM is eliminated, further Cas9-induced DSB formation is prevented. This allows for the construction of cells with small random deletions or insertions, specific mutations and small or large insertions (DiCarlo et al., 2013; Bao et al., 2015; Ryan et al., 2014).

Several CRISPR/Cas9 plasmid systems have been reported for engineering the *S. cerevisiae* genome. Commonly, Cas9 and gRNA are expressed from separate plasmids, although single-plasmid systems and genomically integrated Cas9 expression cassettes have been reported as well (Laughery et al., 2015; Mans et al., 2015). Cas9 is usually targeted to the nucleus by fusion with a nuclear localization signal (NLS) and encoded on YCp or YEp plasmids. As a too high Cas9 expression level is toxic to yeast cells, inducible or medium-strength constitutive promoters are preferable (Ryan et al., 2014; DiCarlo et al., 2013). The gRNA can be encoded on plasmids (usually YEps) or PCR fragments (DiCarlo et al., 2013; Horwitz et al., 2015). To avoid undesired post-transcriptional modifications of the gRNA and its nuclear export, RNA polymerase III is usually recruited for its transcription. Both small nucleolar RNA (snoRNA) promoters and tRNA promoters have been used to drive gRNA transcription (DiCarlo et al., 2013; Ryan et al., 2014). With the latter approach, higher transcript levels were achieved, which was associated with increased Cas9 cleavage efficiency (Ryan and Cate, 2014). However, a more complex gRNA expression cassette containing a self-cleaving ribozyme was required to avoid the production of tRNA–gRNA fusions.

The CRISPR/Cas system can be used to perform several genome modifications at once. In this case, several gRNAs expression units and donor DNAs need to be supplied (Lee et al., 2015; Horwitz et al., 2015; Jakočiūnas et al., 2015; Ronda et al., 2015). Furthermore, multiple copies of the same DNA sequence can be inserted by targeting abundant genomic sequences, such as the δ sites (Shi et al., 2016). This approach can be combined with the previously described strategies to adjust the copy number, such as poorly expressed dominant marker genes and titration of the selective compound (Lian et al., 2016).

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Summary and outlook

Exploiting the specific properties of different yeast plasmids allows for adjusting the gene dosage. YCps and YEps are maintained extrachromosomally with few or some dozens of plasmids per cell, respectively. YIps usually reside in the host cell genome with a single copy. Special systems and strategies allow copy number tuning for YEps and YIps.

The available yeast vectors represent a solid basis for basic research and biotechnological applications in *S. cerevisiae*. The classical vector systems are complemented by marker-recycling and marker-free integration strategies. Together, these tools allow for an easy and efficient genetic manipulation of laboratory, industrial and wild-type yeast strains.

The ever-decreasing costs for *de novo* DNA synthesis in combination with efficient assembly methods will facilitate the construction of new customized vector systems. Genome engineering tools might substitute classical shuttle vectors in the future in some experiments. However, yeast vectors will remain important tools for example for transient gene delivery in screening experiments and genome engineering.

References

Agaphonov M, Alexandrov A. 2014. Self-excising integrative yeast plasmid vectors containing an intronated recombinase gene. *FEMS Yeast Res* **14**: 1048–1054.

Akada R, Hirosawa I, Kawahata M, *et al.* 2002. Sets of integrating plasmids and gene disruption cassettes containing improved counter-selection markers designed for repeated use in budding yeast. *Yeast* **19**: 393–402.

Akada R, Kitagawa T, Kaneko S, *et al.* 2006. PCR-mediated seamless gene deletion and marker recycling in *Saccharomyces cerevisiae*. *Yeast* **23**: 399–405.

Alani E, Cao L, Kleckner N. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics* **116**: 541–545.

Alberti S, Gitler AD, Lindquist S. 2007. A suite of Gateway cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. *Yeast* **24**: 913–919.

Alexander WG, Doering DT, Hittinger CT. 2014. High-efficiency genome editing and allele replacement in prototrophic and wild strains of *Saccharomyces*. *Genetics* **198**: 859–866.

Alting-Mees MA, Short JM. 1989. pBluescript II: gene mapping vectors. *Nucleic Acids Res* **17**: 9494.

Andrews BJ, McLeod M, Broach J, Sadowski PD. 1986. Interaction of the FLP recombinase of the *Saccharomyces cerevisiae* 2 micron plasmid with mutated target sequences. *Mol Cell Biol* **6**: 2482–2489.

Bach ML, Lacroute F, Botstein D. 1979. Evidence for transcriptional regulation of orotidine-5-phosphate decarboxylase in yeast by hybridization of mRNA to the yeast structural gene cloned in *Escherichia coli*. *Proc Natl Acad Sci U S A* **76**: 386–390.

Bao Z, Xiao H, Liang J, *et al.* 2015. Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. *ACS Synth Biol* **4**: 585–594.

Baudin A, Ozier-Kalogeropoulos O, Denouel A, *et al.* 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **21**: 3329–3330.

Beggs JD. 1978. Transformation of yeast by a replicating hybrid plasmid. *Nature* **275**: 104–109.

Bennett CB, Lewis AL, Baldwin KK, Resnick MA. 1993. Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid. *Proc Natl Acad Sci U S A* **90**: 5613–5617.

Bijovet JF, van der Zanden AL, Goosen N, *et al.* 1991. DNA insertions in the 'silent' regions of the 2 microns plasmid of *Saccharomyces cerevisiae* influence plasmid stability. *Yeast* **7**: 347–356.

Blount BA, Weenink T, Ellis T. 2012. Construction of synthetic regulatory networks in yeast. *FEBS Lett* **586**: 2112–2121.

Boeke JD, LaCroute F, Fink GR. 1984. A positive selection for mutants lacking orotidine-5-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197**: 345–346.

Brachmann CB, Davies A, Cost GJ, *et al.* 1998. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**: 115–132.

Brewer BJ, Fangman WL. 1987. The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell* **51**: 463–471.

Broach JR. 1983. Construction of high copy yeast vectors using 2-microns circle sequences. *Methods Enzymol* **101**: 307–325.

Broach JR, Hicks JB. 1980. Replication and recombination functions associated with the yeast plasmid, 2μ circle. *Cell* **21**: 501–508.

Cakar Z, Sauer U, Bailey J. 1999. Metabolic engineering of yeast: the perils of auxotrophic hosts. *Biotechnol Lett* **21**: 611–616.

Carter Z, Delneri D. 2010. New generation of loxP-mutated deletion cassettes for the genetic manipulation of yeast natural isolates. *Yeast* **27**: 765–775.

Carvalho A, Pereira F, Johansson B. 2013. The MX4blaster cassette: repeated and clean *Saccharomyces cerevisiae* genome modification using the genome-wide deletion collection. *FEMS Yeast Res* **13**: 711–719.

Chan KM, Liu YT, Ma CH, *et al.* 2013. The 2 micron plasmid of *Saccharomyces cerevisiae*: a miniaturized selfish genome with optimized functional competence. *Plasmid* **70**: 2–17.

Chattoo BB, Sherman F, Azubalis DA, *et al.* 1979. Selection of lys2 mutants of the yeast *Saccharomyces cerevisiae* by the utilization of alpha-aminoadipate. *Genetics* **93**: 51–65.

Chee MK, Haase SB. 2012. New and redesigned pRS plasmid shuttle vectors for genetic manipulation of *Saccharomyces cerevisiae*. *G3 (Bethesda)* **2**: 515–526.

Chen Y, Partow S, Scalcinati G, *et al.* 2012. Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production. *FEMS Yeast Res* **12**: 598–607.

Chinery SA, Hinchliffe E. 1989. A novel class of vector for yeast transformation. *Curr Genet* **16**: 21–25.

Chlebowicz-Sledziewska E, Sledziewski AZ. 1985. Construction of multicopy yeast plasmids with regulated centromere function. *Gene* **39**: 25–31.

Christianson TW, Sikorski RS, Dante M, *et al.* 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119–122.

Clarke L, Carbon J. 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* **287**: 504–509.

Clark-Walker GD, Miklos GL. 1974. Localization and quantification of circular DNA in yeast. *Eur J Biochem* **41**: 359–365.

Compagno C, Tura A, Ranzi B, *et al.* 1993. Copy number modulation in an autoselection system for stable plasmid maintenance in *Saccharomyces cerevisiae*. *Biotechnol Prog* **9**: 594–599.

Da Silva NA, Srikrishnan S. 2012. Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **12**: 197–214.

Davidson JF, Schiestl RH. 2000. Mis-targeting of multiple gene disruption constructs containing his G. *Curr Genet* **38**: 188–190.

del Solar G, Giraldo R, Ruiz-Echevarria MJ, *et al.* 1998. Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**: 434–464.

Delneri D, Tomlin GC, Wixon JL, *et al.* 2000. Exploring redundancy in the yeast genome: an improved strategy for use of the cre-loxP system. *Gene* **252**: 127–135.

DiCarlo JE, Norville JE, Mali P, *et al.* 2013. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res* **41**: 4336–4343.

Dobson MJ, Yull FE, Molina M, *et al.* 1988. Reconstruction of the yeast 2 micron plasmid partitioning mechanism. *Nucleic Acids Res* **16**: 7103–7117.

Eibel H, Philippsen P. 1983. Identification of the cloned *S. cerevisiae* LYS2 gene by an integrative transformation approach. *Mol Gen Genet* **191**: 66–73.

Erhart E, Hollenberg CP. 1983. The presence of a defective LEU2 gene on 2μ DNA recombinant plasmids of *Saccharomyces cerevisiae* is responsible for curing and high copy number. *J Bacteriol* **156**: 625–635.

Eriksson P, Thomas LR, Thorburn A, Stillman DJ. 2004. pRS yeast vectors with a LYS2 marker. *Biotechniques* **36**: 212–213.

Fairhead C, Dujon B. 1993. Consequences of unique double-stranded breaks in yeast chromosomes: death or homozygosis. *Mol Gen Genet* **240**: 170–178.

Fang F, Salmon K, Shen MWY, *et al.* 2011. A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. *Yeast* **28**: 123–136.

Fangman WL, Hice RH, Chlebowicz-Sledziewska E. 1983. ARS replication during the yeast S phase. *Cell* **32**: 831–838.

Frazer LN, O'Keefe RT. 2007. A new series of yeast shuttle vectors for the recovery and identification of multiple plasmids from *Saccharomyces cerevisiae*. *Yeast* **24**: 777–789.

Fujii T, Kondo K, Shimizu F, *et al.* 1990. Application of a ribosomal DNA integration vector in the construction of a brewer's yeast having alpha-acetolactate decarboxylase activity. *Appl Environ Microbiol* **56**: 997–1003.

Futcher AB. 1986. Copy number amplification of the 2 micron circle plasmid of *Saccharomyces cerevisiae*. *J Theor Biol* **119**: 197–204.

Futcher B, Carbon J. 1986. Toxic effects of excess cloned centromeres. *Mol Cell Biol* **6**: 2213–2222.

Futcher AB, Cox BS. 1983. Maintenance of the 2 microns circle plasmid in populations of *Saccharomyces cerevisiae*. *J Bacteriol* **154**: 612–622.

Futcher AB, Cox BS. 1984. Copy number and the stability of 2-micron circle-based artificial plasmids of *Saccharomyces cerevisiae*. *J Bacteriol* **157**: 283–290.

Gerbaud C, Guerineau M. 1980. 2 micron plasmid copy number in different yeast strains and repartition of endogenous and 2 micron chimeric plasmids in transformed strains. *Curr Genet* **1**: 219–228.

Geymonat M, Spanos A, Sedgwick SG. 2007. A *Saccharomyces cerevisiae* autoselection system for optimised recombinant protein expression. *Gene* **399**: 120–128.

Giaever G, Chu AM, Ni L, *et al.* 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**: 387–391.

Gietz RD, Sugino A. 1988. New yeast–*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**: 527–534.

Gnügge R, Liphardt T, Rudolf F. 2016. A shuttle vector series for precise genetic engineering of *Saccharomyces cerevisiae*. *Yeast* **33**: 83–98.

Goldstein AL, McCusker JH. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541–1553.

Gritz L, Davies J. 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**: 179–188.

Gueldener U, Heinisch J, Koehler GJ, *et al.* 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* **30**: e23.

Guerineau M, Grandchamp C, Paoletti C, Slonimski P. 1971. Characterization of a new class of circular DNA molecules in yeast. *Biochem Biophys Res Commun* **42**: 550–557.

Hadfield C, Cashmore AM, Meacock PA. 1986. An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. *Gene* **45**: 149–158.

Hajra S, Ghosh SK, Jayaram M. 2006. The centromere-specific histone variant Cse4p (CENP-A) is essential for functional chromatin architecture at the yeast 2-μm circle partitioning locus and promotes equal plasmid segregation. *J Cell Biol* **174**: 779–790.

Hartley JL, Donelson JE. 1980. Nucleotide sequence of the yeast plasmid. *Nature* **286**: 860–865.

Hicks JB, Hinnen A, Fink GR. 1979. Properties of yeast transformation. *Cold Spring Harb Symp Quant Biol* **43**(Pt 2): 1305–1313.

Hieter P, Mann C, Snyder M, Davis RW. 1985. Mitotic stability of yeast chromosomes: a colony colour assay that measures nondisjunction and chromosome loss. *Cell* **40**: 381–392.

Hill A, Bloom K. 1987. Genetic manipulation of centromere function. *Mol Cell Biol* **7**: 2397–2405.

Hill JE, Myers AM, Koerner TJ, Tzagoloff A. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**: 163–167.

Hinnen A, Hicks JB, Fink GR. 1978. Transformation of yeast. *Proc Natl Acad Sci U S A* **75**: 1929–1933.

Horwitz A, Walter J, Schubert M, *et al.* 2015. Efficient multiplexed integration of synergistic alleles and metabolic pathways in yeasts via CRISPR-Cas. *Cell Systems* **1**: 88–96.

Huberman JA, Spotila LD, Nawotka KA, *et al.* 1987. The in vivo replication origin of the yeast 2 microns plasmid. *Cell* **51**: 473–481.

Jakočiūnas T, Rajkumar AS, Zhang J, *et al.* 2015. CasEMBLR: Cas9-facilitated multiloci genomic integration of in vivo assembled DNA parts in *Saccharomyces cerevisiae*. *ACS Synth Biol* **4**: 1226–1234.

Jayaram M, Li YY, Broach JR. 1983. The yeast plasmid 2μ circle encodes components required for its high copy propagation. *Cell* **34**: 95–104.

Jensen NB, Strucko T, Kildegaard KR, *et al.* 2014. EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **14**: 238–248.

Jessop-Fabre MM, Jakočiūnas T, Stovicek V, *et al.* 2016. EasyClone-Marker Free: a vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR- Cas9. *Biotechnol J* **11**: 1110–1117.

Jiang W, Marraffini LA. 2015. CRISPR-Cas: new tools for genetic manipulations from bacterial immunity systems. *Annu Rev Microbiol* **69**: 209–228.

Jimenez A, Davies J. 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. *Nature* **287**: 869–871.

Jinek M, Chylinski K, Fonfara I, *et al.* 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816–821.

Johansson B, Hahn-Haegerdal B. 2002. Overproduction of pentose phosphate pathway enzymes using a new CRE-loxP expression vector for repeated genomic integration in *Saccharomyces cerevisiae*. *Yeast* **19**: 225–231.

Kaneko S, Tanaka T, Noda H, *et al.* 2009. Marker-disruptive gene integration and URA3 recycling for multiple gene manipulation in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **83**: 783–789.

Karim AS, Curran KA, Alper HS. 2013. Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications. *FEMS Yeast Res* **13**: 107–116.

Kato H, Matsuda F, Yamada R, *et al.* 2013. Cocktail δ-integration of xylose assimilation genes for efficient ethanol production from xylose in *Saccharomyces cerevisiae*. *J Biosci Bioeng* **116**: 333–336.

Kawasaki G. 1984. Abstr. P15. In *12th International Conference on Yeast Genetics and Molecular Biology*, Edinburgh, UK.

Kawasaki G, Bell L. 1999. US Patent 5871957.

Khmelinskii A, Meurer M, Duishoev N, *et al.* 2011. Seamless gene tagging by endonuclease-driven homologous recombination. *PLoS One* **6**: 1–8.

Kikuchi Y. 1983. Yeast plasmid requires a cis-acting locus and two plasmid proteins for its stable maintenance. *Cell* **35**: 487–493.

Kim JM, Vanguri S, Boeke JD, *et al.* 1998. Transposable elements and genome organization: a comprehensive survey of retrotransposons revealed by the complete *Saccharomyces cerevisiae* genome sequence. *Genome Res* **8**: 464–478.

Kjeldsen T, Brandt J, Andersen AS, *et al.* 1996. A removable spacer peptide in an alpha-factor-leader/insulin precursor fusion protein improves processing and concomitant yield of the insulin precursor in *Saccharomyces cerevisiae*. *Gene* **170**: 107–112.

Kowalczykowski SC. 2015. An overview of the molecular mechanisms of recombinational DNA repair. *Cold Spring Harb Perspect Biol* **7**: a016410.

Laughery MF, Hunter T, Brown A, *et al.* 2015. New vectors for simple and streamlined CRISPR-Cas9 genome editing in *Saccharomyces cerevisiae*. *Yeast* **32**: 711–720.

Lee FW, Da Silva NA. 1997. Improved efficiency and stability of multiple cloned gene insertions at the delta sequences of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **48**: 339–345.

Lee ME, DeLoache WC, Cervantes B, Dueber JE. 2015. A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synth Biol* **4**: 975–986.

Leite FCB, dos Anjos RSG, Basilio ACM, *et al.* 2013. Construction of integrative plasmids suitable for genetic modification of industrial strains of *Saccharomyces cerevisiae*. *Plasmid* **69**: 114–117.

Lian J, Jin R, Zhao H. 2016. Construction of plasmids with tunable copy numbers in *Saccharomyces cerevisiae* and their applications in pathway optimization and multiplex genome integration. *Biotechnol Bioeng* **113**: 2462–2473.

Loison G, Vidal A, Findeli A, *et al.* 1989. High level of expression of a protective antigen of schistosomes in *Saccharomyces cerevisiae*. *Yeast* **5**: 497–507.

Lopes TS, Klootwijk J, Veenstra AE, *et al.* 1989. High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*: a new vector for high-level expression. *Gene* **79**: 199–206.

Lopes TS, Hakkaart GJ, Koerts BL, *et al.* 1991. Mechanism of high-copy-number integration of pMIRY-type vectors into the ribosomal DNA of *Saccharomyces cerevisiae*. *Gene* **105**: 83–90.

Lorenz MC, Muir RS, Lim E, *et al.* 1995. Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene* **158**: 113–117.

Ma H, Kunes S, Schatz PJ, Botstein D. 1987. Plasmid construction by homologous recombination in yeast. *Gene* **58**: 201–216.

Ma CH, Cui H, Hajra S, *et al.* 2013. Temporal sequence and cell cycle cues in the assembly of host factors at the yeast 2 micron plasmid partitioning locus. *Nucleic Acids Res* **41**: 2340–2353.

Manivasakam P, Weber SC, McElver J, Schiestl RH. 1995. Microhomology mediated PCR targeting in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **23**: 2799–2800.

Mans R, van Rossum HM, Wijsman M, *et al.* 2015. CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **15**: –fov004.

Maury J, Germann SM, Baallal Jacobsen SA, *et al.* 2016. EasyCloneMulti: a set of vectors for simultaneous and multiple genomic integrations in *Saccharomyces cerevisiae*. *PLoS One* **11** .e0150394

Mileyko Y, Joh RI, Weitz JS. 2008. Small-scale copy number variation and large-scale changes in gene expression. *Proc Natl Acad Sci U S A* **105**: 16659–16664.

Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C. 2009. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**: 733–740.

Mumberg D, Mueller R, Funk M. 1994. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22**: 5767–5768.

Mumberg D, Muller R, Funk M. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**: 119–122.

Murray JA, Cesareni G. 1986. Functional analysis of the yeast plasmid partition locus STB. *EMBO J* **5**: 3391–3399.

Murray AW, Szostak JW. 1983. Pedigree analysis of plasmid segregation in yeast. *Cell* **34**: 961–970.

Murray JA, Scarpa M, Rossi N, Cesareni G. 1987. Antagonistic controls regulate copy number of the yeast 2μ plasmid. *EMBO J* **6**: 4205–4212.

Nagels Durand A, Moses T, De Clercq R, *et al.* 2012. A MultiSite Gateway™ vector set for the functional analysis of genes in the model *Saccharomyces cerevisiae*. *BMC Mol Biol* **13**: 30.

Napp S, Da Silva N. 1993. Enhancement of cloned gene product synthesis via autoselection in recombinant *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **41**: 801–810.

Nickoloff JA, Chen EY, Heffron F. 1986. A 24-base-pair DNA sequence from the MAT locus stimulates intergenic recombination in yeast. *Proc Natl Acad Sci U S A* **83**: 7831–7835.

Nour-Eldin HH, Hansen BG, Nørholm MHH, *et al.* 2006. Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments. *Nucleic Acids Res* **34**: e122.

Oldenburg KR, Vo KT, Michaelis S, Paddon C. 1997. Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Res* **25**: 451–452.

Orr-Weaver TL, Szostak JW. 1983. Multiple, tandem plasmid integration in *Saccharomyces cerevisiae*. *Mol Cell Biol* **3**: 747–749.

Orr-Weaver TL, Szostak JW, Rothstein RJ. 1981. Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci U S A* **78**: 6354–6358.

Orr-Weaver TL, Szostak JW, Rothstein RJ. 1983. Genetic applications of yeast transformation with linear and gapped plasmids. *Methods Enzymol* **101**: 228–245.

Ostrov N, Wingler LM, Cornish VW. 2013. Gene assembly and combinatorial libraries in *S. cerevisiae* via reiterative recombination. *Methods Mol Biol* **978**: 187–203.

Parekh RN, Wittrup KD. 1997. Expression level tuning for optimal heterologous protein secretion in *Saccharomyces cerevisiae*. *Biotechnol Prog* **13**: 117–122.

Parekh RN, Shaw MR, Wittrup KD. 1996. An integrating vector for tunable, high copy, stable integration into the dispersed Ty δ sites of *Saccharomyces cerevisiae*. *Biotechnol Prog* **12**: 16–21.

Plessis A, Dujon B. 1993. Multiple tandem integrations of transforming DNA sequences in yeast chromosomes suggest a mechanism for integrative transformation by homologous recombination. *Gene* **134**: 41–50.

Ratzkin B, Carbon J. 1977. Functional expression of cloned yeast DNA in *Escherichia coli*. *Proc Natl Acad Sci U S A* **74**: 487–491.

Ronda C, Maury J, Jakočiūnas T, *et al.* 2015. CrEdit: CRISPR mediated multi-loci gene integration in *Saccharomyces cerevisiae*. *Microb Cell Fact* **14**: 97.

Rose AB, Broach JR. 1990. Propagation and expression of cloned genes in yeast: 2-microns circle-based vectors. *Methods Enzymol* **185**: 234–279.

Rothstein RJ. 1983. One-step gene disruption in yeast. *Methods Enzymol* **101**: 202–211.

Rothstein R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* **194**: 281–301.

Ruff P, Koh KD, Keskin H, *et al.* 2014. Aptamer-guided gene targeting in yeast and human cells. *Nucleic Acids Res* **42**: e61.

Runge KW, Wellinger RJ, Zakian VA. 1991. Effects of excess centromeres and excess telomeres on chromosome loss rates. *Mol Cell Biol* **11**: 2919–2928.

Russell PR. 1985. Transcription of the triose-phosphate-isomerase gene of *Schizosaccharomyces pombe* initiates from a start point different from that in *Saccharomyces cerevisiae*. *Gene* **40**: 125–130.

Ryan OW, Cate JHD. 2014. Multiplex engineering of industrial yeast genomes using CRISPRm. *Methods Enzymol* **546**: 473–489.

Ryan OW, Skerker JM, Maurer MJ, *et al.* 2014. Selection of chromosomal DNA libraries using a multiplex crispr system. *Elife* **3** .e03703

Sakai A, Shimizu Y, Hishinuma F. 1990. Integration of heterologous genes into the chromosome of *Saccharomyces cerevisiae* using a delta sequence of yeast retrotransposon Ty. *Appl Microbiol Biotechnol* **33**: 302–306.

Sakai A, Ozawa F, Higashizaki T, *et al.* 1991. Enhanced secretion of human nerve growth factor from *Saccharomyces cerevisiae* using an advanced δ-integration system. *Nat Biotechnol* **9**: 1382–1385.

Sauer B. 1987. Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**: 2087–2096.

Scott-Drew S, Murray JA. 1998. Localisation and interaction of the protein components of the yeast 2μ circle plasmid partitioning system suggest a mechanism for plasmid inheritance. *J Cell Sci* **111**(Pt 13): 1779–1789.

Shen MWY, Fang F, Sandmeyer S, Da Silva NA. 2012. Development and characterization of a vector set with regulated promoters for systematic metabolic engineering in *Saccharomyces cerevisiae*. *Yeast* **29**: 495–503.

Shi S, Liang Y, Zhang MM, *et al.* 2016. A highly efficient single-step, markerless strategy for multi-copy chromosomal integration of large biochemical pathways in *Saccharomyces cerevisiae*. *Metab Eng* **33**: 19–27.

Short JM, Fernandez JM, Sorge JA, Huse WD. 1988. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res* **16**: 7583–7600.

Shusta EV, Raines RT, Pluecthun A, Wittrup KD. 1998. Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nat Biotechnol* **16**: 773–777.

Siddiqui MS, Choksi A, Smolke CD. 2014. A system for multilocus chromosomal integration and transformation-free selection marker rescue. *FEMS Yeast Res* **14**: 1171–1185.

Siewers V. 2014. An overview on selection marker genes for transformation of *Saccharomyces cerevisiae*. *Methods Mol Biol* **1152**: 3–15.

Sikorski RS, Boeke JD. 1991. In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Methods Enzymol* **194**: 302–318.

Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.

Simchen G, Winston F, Styles CA, Fink GR. 1984. Ty-mediated gene expression of the LYS2 and HIS4 genes of *Saccharomyces cerevisiae* is controlled by the same SPT genes. *Proc Natl Acad Sci U S A* **81**: 2431–2434.

Singh A, Sherman F. 1974. Association of methionine requirement with methyl mercury resistant mutants of yeast. *Nature* **247**: 227–229.

Skryabin KG, Eldarov MA, Larionov VL, *et al.* 1984. Structure and function of the nontranscribed spacer regions of yeast rDNA. *Nucleic Acids Res* **12**: 2955–2968.

So M, Gill R, Falkow S. 1975. The generation of a ColE1-Apr cloning vehicle which allows detection of inserted DNA. *Mol Gen Genet* **142**: 239–249.

Solis-Escalante D, Kuijpers NGA, Bongaerts N, *et al.* 2013. amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. *FEMS Yeast Res* **13**: 126–139.

Solis-Escalante D, Kuijpers NGA, van der Linden FH, *et al.* 2014. Efficient simultaneous excision of multiple selectable marker cassettes using I-SceI-induced double-strand DNA breaks in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **14**: 741–754.

Som T, Armstrong KA, Volkert FC, Broach JR. 1988. Autoregulation of 2 micron circle gene expression provides a model for maintenance of stable plasmid copy levels. *Cell* **52**: 27–37.

Stevens BJ, Moustacchi E. 1971. Gamma satellite DNA and small twisted circular molecules in yeast *Saccharomyces cerevisiae*. *Exp Cell Res* **64**: 259–266.

Stinchcomb DT, Struhl K, Davis RW. 1979. Isolation and characterisation of a yeast chromosomal replicator. *Nature* **282**: 39–43.

Storici F, Coglievina M, Bruschi CV. 1999. A 2-μm DNA-based marker recycling system for multiple gene disruption in the yeast *Saccharomyces cerevisiae*. *Yeast* **15**: 271–283.

Storici F, Lewis LK, Resnick MA. 2001. In vivo site-directed mutagenesis using oligonucleotides. *Nat Biotechnol* **19**: 773–776.

Storici F, Durham CL, Gordenin DA, Resnick MA. 2003. Chromosomal site-specific double-strand breaks are efficiently targeted for repair by oligonucleotides in yeast. *Proc Natl Acad Sci U S A* **100**: 14994–14999.

Stotz A, Linder P. 1990. The ADE2 gene from *Saccharomyces cerevisiae*: sequence and new vectors. *Gene* **95**: 91–98.

Struhl K, Davis RW. 1977. Production of a functional eukaryotic enzyme in *Escherichia coli*: cloning and expression of the yeast structural gene for imidazole-glycerolphosphate dehydratase (his3). *Proc Natl Acad Sci U S A* **74**: 5255–5259.

Struhl K, Stinchcomb DT, Scherer S, Davis RW. 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. *Proc Natl Acad Sci U S A* **76**: 1035–1039.

Svetec IK, Stafa A, Zgaga Z. 2007. Genetic side effects accompanying gene targeting in yeast: the influence of short heterologous termini. *Yeast* **24**: 637–652.

Symington LS, Rothstein R, Lisby M. 2014. Mechanisms and regulation of mitotic recombination in *Saccharomyces cerevisiae*. *Genetics* **198**: 795–835.

Szostak JW, Wu R. 1979. Insertion of a genetic marker into the ribosomal DNA of yeast. *Plasmid* **2**: 536–554.

Taxis C, Knop M. 2006. System of centromeric, episomal, and integrative vectors based on drug resistance markers for *Saccharomyces cerevisiae*. *Biotechniques* **40**: 73–78.

Thim L, Hansen MT, Norris K, *et al.* 1986. Secretion and processing of insulin precursors in yeast. *Proc Natl Acad Sci U S A* **83**: 6766–6770.

Thomas BJ, Rothstein R. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell* **56**: 619–630.

Toyn JH, Gunyuzlu PL, White WH, *et al.* 2000. A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. *Yeast* **16**: 553–560.

Tschumper G, Carbon J. 1983. Copy number control by a yeast centromere. *Gene* **23**: 221–232.

Ullmann A, Jacob F, Monod J. 1967. Characterization by in vitro complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. *J Mol Biol* **24**: 339–343.

Unternahrer S, Pridmore D, Hinnen A. 1991. A new system for amplifying 2 microns plasmid copy number in *Saccharomyces cerevisiae*. *Mol Microbiol* **5**: 1539–1548.

Velmurugan S, Yang XM, Chan CS, *et al.* 2000. Partitioning of the 2-μm circle plasmid of *Saccharomyces cerevisiae*: functional coordination with chromosome segregation and plasmid-encoded rep protein distribution. *J Cell Biol* **149**: 553–566.

Vickers CE, Bydder SF, Zhou Y, Nielsen LK. 2013. Dual gene expression cassette vectors with antibiotic selection markers for engineering in *Saccharomyces cerevisiae*. *Microb Cell Fact* **12**: 96.

Vieira J, Messing J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259–268.

Volkert FC, Broach JR. 1986. Site-specific recombination promotes plasmid amplification in yeast. *Cell* **46**: 541–550.

Voth WP, Richards JD, Shaw JM, Stillman DJ. 2001. Yeast vectors for integration at the HO locus. *Nucleic Acids Res* **29**: E59–E59.

Wach A, Brachat A, Poehlmann R, Philippsen P. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793–1808.

Wach A, Brachat A, Alberti-Segui C, *et al.* 1997. Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast* **13**: 1065–1075.

Wang X, Wang Z, Da Silva NA. 1996. G418 Selection and stability of cloned genes integrated at chromosomal δ sequences of *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **49**: 45–51.

Weber E, Engler C, Gruetzner R, *et al.* 2011. A modular cloning system for standardized assembly of multigene constructs. *PLoS One* **6** .e16765

Weinstock KG, Strathern JN. 1993. Molecular genetics in *Saccharomyces kluyveri*: the HIS3 homolog and its use as a selectable marker gene in *S. kluyveri and Saccharomyces cerevisiae*. *Yeast* **9**: 351–361.

Westermann S, Drubin DG, Barnes G. 2007. Structures and functions of yeast kinetochore complexes. *Annu Rev Biochem* **76**: 563–591.

Wilson JH, Leung WY, Bosco G, *et al.* 1994. The frequency of gene targeting in yeast depends on the number of target copies. *Proc Natl Acad Sci U S A* **91**: 177–181.

Wingler LM, Cornish VW. 2011. Reiterative Recombination for the in vivo assembly of libraries of multigene pathways. *Proc Natl Acad Sci U S A* **108**: 15135–15140.

Woolford JL, Jr, Baserga SJ. 2013. Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. *Genetics* **195**: 643–681.

Wosika V, Durandau E, Varidel C, *et al.* 2016. New families of single integration vectors and gene tagging plasmids for genetic manipulations in budding yeast. *Mol Genet Genomics* **291**: 2231–2240.

Xie W, Liu M, Lv X, *et al.* 2014. Construction of a controllable β-carotene biosynthetic pathway by decentralized assembly strategy in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **111**: 125–133.

Yamada R, Taniguchi N, Tanaka T, *et al.* 2010. Cocktail δ-integration: a novel method to construct cellulolytic enzyme expression ratio-optimized yeast strains. *Microb Cell Fact* **9**: 32.

Zakian VA, Brewer BJ, Fangman WL. 1979. Replication of each copy of the yeast 2 micron DNA plasmid occurs during the S phase. *Cell* **17**: 923–934.