Yeast
Yeast 2010; **27**: 189–195.
Published online 10 December 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1741

Research Article

A novel kanamycin/G418 resistance marker for direct selection of transformants in *Escherichia coli* and different yeast species

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Received: 12 August 2009
Accepted: 31 October 2009

Abstract

We have developed a set of cloning vectors possessing a modified Tn903 kanamycin resistance gene that enables the selection of both kanamycin-resistant transformants in *Escherichia coli* and G418-resistant transformants in the yeasts *Saccharomyces cerevisiae, Hansenula polymorpha* and *Pichia pastoris*. Expression of this gene in yeast is controlled by the *H. polymorpha* glyceraldehyde-3-phosphate dehydrogenase promoter, while expression in *E. coli* is governed by an upstream *E. coli lacZ* promoter. Applicability of the vectors for gene disruption in *H. polymorpha* and *S. cerevisiae* was demonstrated by inactivation of the *HpMAL1* and *URA3* genes, respectively. One of the vectors possesses a *H. polymorpha ARS* allowing plasmid maintenance in an episomal state. The small size of the vectors (2–2.5 kb) makes them convenient for routine DNA cloning. In addition, we report a novel approach for construction of gene disruption cassettes.

Keywords: antibiotic resistance; transformation; gene disruption; cloning vector; yeast

Introduction

Genetic manipulations often require introduction of recombinant DNA constructs into cells and subsequent selection of transformants, which is ensured by the use of an appropriate plasmid selectable marker. Yeast recipient strains usually possess auxotrophic mutations, which can be complemented by the corresponding chromosomal genes introduced into genetic constructs as selectable markers. However, heterologous genes conferring resistance to some antibiotics (e.g. G418, hygromycin and Zeocin) can also be used as plasmid selectable markers (Davies and Jimenez, 1980; Gritz and Davies, 1983; Gatignol *et al*., 1987). Such markers are especially helpful if the host strain does not possess appropriate auxotrophic mutations, or if these mutations are required for subsequent genetic manipulations.

As a rule, creating a genetic construct for introduction into yeast cells involves an intermediate step of plasmid amplification in *Escherichia coli*. The use of markers that function in both *E. coli* and yeast may reduce the size of plasmid and simplify its modifications. A set of vectors possessing a Zeocin resistance marker that works in both *E. coli* and yeast has been developed (Invitrogen). However, the high cost of Zeocin restricts extensive use of plasmids with this marker. Here, we describe a novel selectable marker providing resistance to kanamycin in *E. coli* and to G418 in the yeasts *Saccharomyces cerevisiae, Hansenula polymorpha* and *Pichia pastoris*.

Materials and methods

Strains, media and culture conditions

Standard YPD (2% peptone, 1% yeast extract, 2% glucose) and SD (0.67% yeast nitrogen base, 2% glucose) media were used to cultivate yeast cells. *E. coli* was cultivated on 2YT medium (1% yeast extract, 1.6% tryptone, 0.5% NaCl) supplemented with 50 mg/l kanamycin. *H. polymorpha* DL1-L (*leu2*) (Sohn *et al*., 1996), *S. cerevisiae* 10B-H49 (*MATα kar1-1 ade2-1 leu1 SUQ5 his3 lys1*) (Ter-Avanesyan *et al*., 1994), *P. pastoris* GS115 (*his4*) (Cregg *et al*., 1985), and *E. coli* DH5α (Woodcock *et al*., 1989) strains were used in this work. Yeasts were transformed according to the standard procedure (Ito *et al*., 1983), with modifications for *H. polymorpha* (Bogdanova *et al*., 1995) and *P. pastoris* (Cregg and Russell, 1998). *E. coli* was transformed as previously described (Inoue *et al*., 1990). YPD supplemented with 200 mg/l G418 was used for selection of G418-resistant transformants of *H. polymorpha* and *P. pastoris*, while the *S. cerevisiae* transformants were selected on 150 mg/l G418. Prior to plating onto G418-containing medium, yeast transformation mixes were pre-incubated in liquid YPD for 1 h. *S. cerevisiae* and *P. pastoris* cells were cultivated at 30 °C, while *H. polymorpha* was grown at 37 °C.

Plasmid construction

Routine *in vitro* DNA manipulations were carried out as previously described (Sambrook *et al*., 1989). The pKAM444 plasmid was obtained in two steps (Figure 1). In the first step, the region of the pUK21 plasmid (Vieira and Messing, 1990) between the *Bsp*HI site 51 bp upstream from the aminoglycoside 3′-phosphotransferase (*APH*) open reading frame (ORF) and the *Xba*I site of the polylinker was replaced with the 0.6 kb *Xba*I–*Eco*RI fragment of pGAG418 (Sohn *et al*., 1999) bearing *H. polymorpha* glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAPDH}). Prior to ligation, the *Eco*RI- and *Bsp*HI-generated termini of the insert and vector, respectively, were treated with Mung Bean Nuclease. None of the *E. coli* transformants obtained with the ligation mix possessed the correctly assembled fragments, indicating that the resulting selectable marker might not be efficiently expressed in *E. coli*. The plasmid pKNR80, which was finally selected at this step, possessed deletions at the junction of P_{GAPDH} and *APH* extending for 44 bp towards the *APH* ORF and 12 bp towards P_{GAPDH}, which apparently led to an improvement of this region for *APH* expression in *E. coli*. The *Xba*I–*Spe*I fragment of pKNR80 was then deleted to obtain pKAM444. To construct the pKAM540 plasmid for

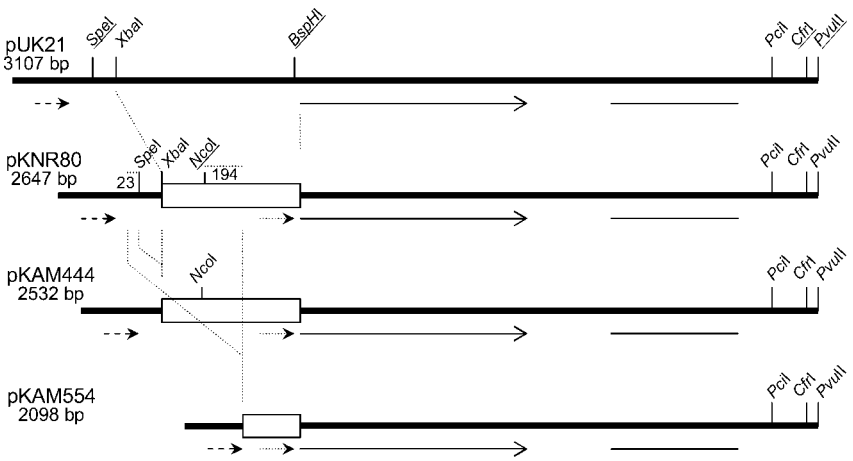

Figure 1. Construction scheme for the pKNR80, pKAM444 and pKAM554 plasmids. Sequences originating from the pUK21 plasmid are designated by the thick line; the DNA fragments of the P_{GAPDH} chromosomal locus are designated by the open bars; position of *lacZ* promoter, P_{GAPDH}, and *APH* ORF are designated by dashed, dotted and solid arrows, respectively; position of *E. coli* replication origin is indicated by the thin line. Restriction sites that are not unique in the plasmid are underlined. Numbers 23 and 194 under the dotted lines indicate the size (bp) of *BAL31* deletions obtained during the construction of the pKAM554 plasmid. Vertical dotted lines indicate sequence correspondence in parent and descendant molecules

DOI: 10.1002/yea

HpMAL1 disruption (Figure 2), the *H. polymorpha* chromosomal DNA was digested with *Ecl*136II, self-ligated and used as a template for PCR with the primers U (5′-cattc aacga gactg agcac-3′) and L (5′-aggaa ttaat attgt caaga ggg-3′). Then, the obtained PCR product was inserted between the *Pci*I and *Pvu*II sites of pKAM444. To obtain pKAM554, the pKNR80 plasmid was cleaved with *Spe*I and *Nco*I, treated with BAL31 and self-ligated (Figure 1). pKAM555 (Figure 3) was constructed by replacing the *Pci*I–*Cfr*I fragment in pKAM554 with the DNA fragment obtained by annealing of the oligonucleotides 5′-catga catgg cgcca tggga agctt ggatc cccgg gtacc gagct cgaat tcact-3′ and 5′-ggcca gtgaa ttcga gctcg gtacc cgggg atcca agctt cccat ggcgc catgt-3′. To obtain the pKAM567A and pKAM567B plasmids (Figure 3), a linker obtained by annealing of the oligonucleotides 5′-gatcc aagct tccca tggcg ccatg tcatg agtggc-3′ and 5′-gccact catga catgg cgcca tggga agctt ggatc-3′ was introduced into the *Pvu*II site of the pKAM554 plasmid in two opposite orientations. The pKAM556 plasmid (Figure 3) was constructed by replacing the *Kpn*I–*Pvu*II fragment of pKAM555 with the *HARS6*-bearing *Kpn*I–*Eco*RV fragment of the p2CHA6 plasmid (Chechenova *et al*., 2004). To obtain the pKAM558 plasmid, the *HpLEU2*-containing *Sal*I–*Bam*HI fragment of the pCLHX plasmid (Sohn *et al*., 1996) was introduced between the *Xho*I and *Bam*HI sites of pKAM556. The pESC1 plasmid was constructed by insertion of the 248 bp *Eco*RV–*Stu*I fragment of the *S. cerevisiae URA3* gene into the *Pvu*II site of pKAM444.

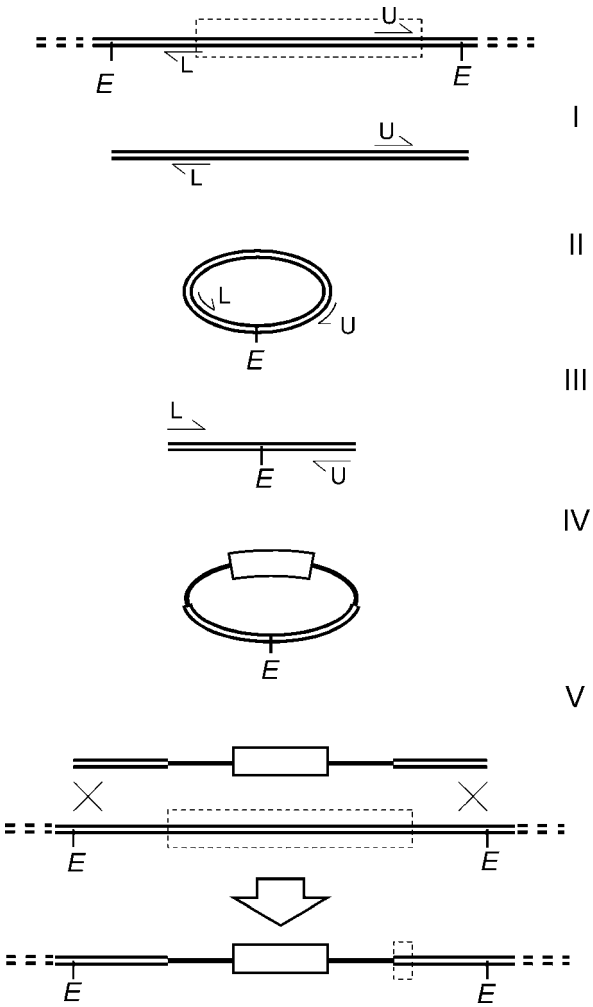

Figure 2. Construction scheme for the *HpMAL1* disruption cassette. (I) *H. polymorpha* chromosomal DNA was digested with *Ecl*136II; (II) restriction fragments were self-ligated; and (III) were used as a template for PCR with primers U and L; (IV) PCR product was inserted into the pKAM444 plasmid; (V) the resulting pKAM540 plasmid was linearized with *Ecl*136II and used for *H. polymorpha* transformation. Recombination of the linearized plasmid with the chromosomal *HpMAL1* gene resulted in replacement of its internal sequence with the sequence of pKAM444 plasmid. Double lines, *H. polymorpha* chromosomal sequences; single lines, pKAM444 sequence; empty bar, selectable marker; E, *Ecl*136II restriction sites; U and L, primers. The dashed frames mark sequences of the *HpMAL1* ORF

Results and discussion

pKAM444 cloning vector

The original aim of this work was to develop a convenient vector with a G418 resistance marker for *H. polymorpha* transformation. The marker, which was previously constructed for this purpose (Sohn *et al*., 1999), consisted of the P_{GAPDH}-controlled Tn903 ORF encoding aminoglycoside 3′-phosphotransferase (*APH*). Plasmids equipped with the bacterial ampicillin resistance marker and containing the aforementioned P_{GAPDH}–*APH* gene provided *E. coli* transformants selected on ampicillin-containing medium with kanamycin resistance, but the primary selection of transformants on kanamycin plates was not possible (data not shown). Another disadvantage of this marker was the presence of the *Hin*dIII, *Sma*I and *Xho*I restriction sites within its ORF. Notably, all

DOI: 10.1002/yea

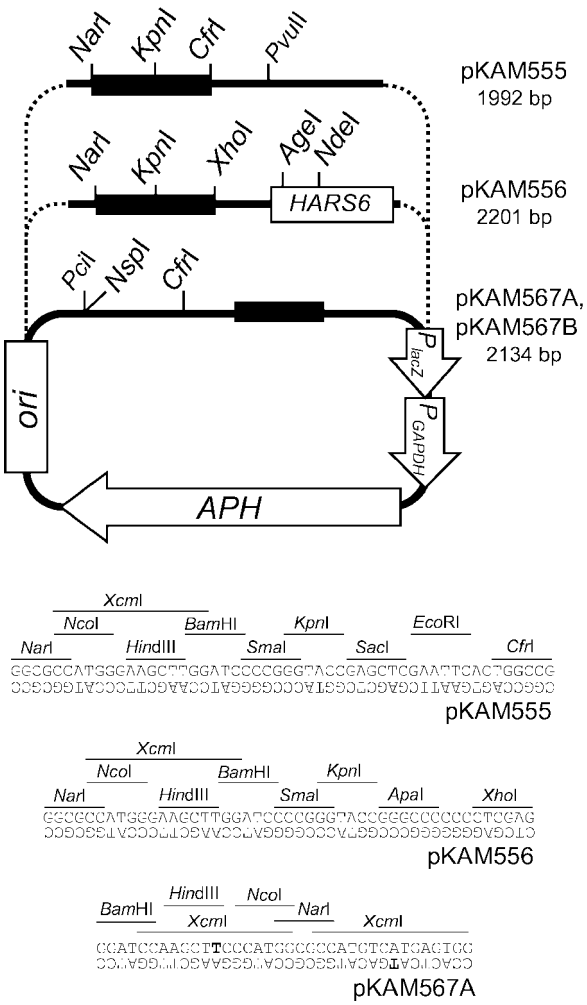

Figure 3. Schemes of the pKAM555, pKAM556, pKAM567A and pKAM567B plasmids. Positions of polylinkers on the schemes are indicated by solid bars; their nucleotide sequences are presented below the schemes. The pKAM567B polylinker sequence is identical to that of pKAM567A but has the reverse orientation. The T residues representing 3′ overhangs after digestion of the pKAM567A with *Xcm*I are shown in bold

these restriction sites had been removed from the kanamycin resistance marker in the pUK21 cloning vector (Vieira and Messing, 1990).

To overcome the disadvantages of the previous marker, the pKAM444 plasmid was constructed (Figure 1). This plasmid possessed pUK21 *APH* ORF fused to P_{GAPDH} for its efficient expression in yeast. Expression in *E. coli* was ensured by the *lacZ* promoter (data not shown), located upstream of P_{GAPDH}, and by a favourable sequence at the junction of P_{GAPDH} and *APH* ORF randomly selected during the plasmid construction (see Materials and methods).

To confirm the ability of pKAM444 to confer G418 resistance in yeast, *H. polymorpha* DL1-L and *P. pastoris* GS155 were transformed with this plasmid, which was linearized by *Pvu*II prior to transformation. In *H. polymorpha*, linearization of transforming DNA stimulates its integration into random genomic sites (van Dijk *et al.*, 2000) and one could expect a similar effect in the related yeast, *P. pastoris*. Indeed, transformations of both species produced a number of G418-resistant clones. The presence of the plasmid sequence in transformants was confirmed by PCR analysis (data not shown). Efficient transformation of *P. pastoris* with the plasmid that did not contain any its DNA sequences indicates the random integration of the plasmid.

In contrast to *H. polymorpha* and *P. pastoris*, integration of transforming DNA into the *S. cerevisiae* genome occurs mainly via homologous recombination. To study whether the obtained marker could be used for transformation of *S. cerevisiae*, the integrative vector pESC1 was constructed by insertion of an internal fragment of the *S. cerevisiae URA3* ORF into the pKAM444 plasmid. Integration of the pESC1 plasmid by a single cross-over into the chromosomal *URA3* locus should generate two defective copies of this gene. To stimulate recombination, prior to the transformation this plasmid was cleaved by *Sca*I within the *URA3* sequence. All transformants of the 10B-H49 strain obtained on G418 plates were unable to grow on uracil omission medium, indicating that the chromosomal *URA3* gene was disrupted.

Application of the pKAM444 plasmid for gene replacement

To demonstrate applicability of the obtained selectable marker for gene disruption in *H. polymorpha*, plasmid pKAM540 (see Materials and methods), bearing the disruption cassette for the maltase-encoding gene *HpMAL1* (Liiv *et al.*, 2001), was constructed. The routine approach for the disruption of chromosomal genes by replacement with a selectable marker includes transformation of cells with a disruption cassette — a linear DNA fragment containing a selectable marker flanked by sequences of a target locus. The classical procedure for obtaining such gene disruption cassettes

DOI: 10.1002/yea

consists of two cloning steps: (a) cloning of a target locus sequence in an *E. coli* vector; and (b) replacement of the internal part of the gene being disrupted with a selectable marker. Prior to yeast transformation, the disruption cassette is excised from the plasmid by restriction enzymes. Here, an alternative approach including only one cloning step was used to construct the *HpMAL1* disruption cassette (Figure 2). In this approach, the PCR product obtained by reverse amplification of the flanking (RAF) homology sequences is cloned into a vector equipped with a yeast selectable marker. The entire plasmid then serves as a disruption cassette. This allows the use of a single selectable marker for transformation of both *E. coli* and yeast. To disrupt the *HpMAL1* gene, the strain DL1-L was transformed with the linearized pKAM540 plasmid (Figure 2V). Approximately 60% of the *H. polymorpha* transformants selected on G418-containing medium were unable to grow on a medium containing maltose as a sole carbon source, indicating disruption of the *MAL1* gene.

pKAM554, pKAM555, pKAM556, pKAM567A and pKAM567B vectors

The fragment of the P_{GAPDH} locus in the pKAM444 plasmid begins from the −578 position, counting from the GAPDH ORF (Sohn *et al*., 1999), and contains several restriction sites, which could be undesirable for further use of the plasmid as a cloning vector. It has been previously shown that even the 146 bp P_{GAPDH} fragment lacking the restriction sites mentioned above provides *APH* expression at levels sufficient for high resistance of the *H. polymorpha* transformants to G418 (Sohn *et al*., 1999). Based on this, the region bearing the undesirable restriction sites was removed during construction of the pKAM554 plasmid (Figure 1).

Like pKAM444, the pKAM554 plasmid possessed only *Pci*I, *Cfr*I and *Pvu*II sites suitable for cloning of DNA fragments. To make this vector more convenient, a linker possessing several other restriction sites was introduced between the *Pci*I and *Cfr*I sites of pKAM554 (Figure 1) and the obtained plasmid was designated pKAM555 (Figure 3). The pKAM556 plasmid (Figure 3) was obtained from pKAM555 by the insertion of *HARS6* for autonomous replication in *H. polymorpha* cells. Importantly, two additional unique restriction sites (*Apa*I and *Xho*I) were introduced into the polylinker together with *HASR6*. Along with the restriction sites in the polylinker, *Age*I and *Nde*I within *HARS6* (Figure 3) could also be used as cloning sites if functional *HARS6* is not required.

The growth rates of *E. coli* transformants with the obtained plasmids on kanamycin-containing medium and plasmid DNA yields from equal volumes of overnight cultures were even increased compared to that of transformants with the original vector pUK21 (data not shown), indicating that plasmid maintenance in *E. coli* cells was not compromised.

According to our experience, insertion of certain DNA fragments (e.g. the *Xho*II–*Xho*II fragment of the *HpLEU2* locus) into the pUK21 polylinker inhibits growth of corresponding bacterial transformants on kanamycin-containing medium. In contrast, insertion of this *HpLEU2*-containing fragment into the polylinker of pKAM556 did not presently affect growth of *E. coli* transformants or the plasmid DNA yield (data not shown).

The pKAM556 plasmid was equipped with a *HARS* to allow its use as an episomal *H. polymorpha* vector. To confirm the ability of this plasmid to replicate autonomously, the *H. polymorpha* DL1-L strain (*leu2*) was transformed with the pKAM556-based pKAM558 plasmid bearing the *HpLEU2* marker, which was required to facilitate monitoring of plasmid mitotic stability. Transformation frequencies with this plasmid upon selection for G418 resistance and for leucin prototrophy were the same. The Leu^+ phenotype of nine tested G418-resistant transformants was mitotically unstable, indicating the episomal state of the plasmid. Along with the pKAM555 and pKAM556 plasmids, two additional derivatives of the pKAM554 plasmid, pKAM567A and pKAM567B, possessing another set of cloning sites (Figure 3), were constructed. Cleavage of these plasmids by *Xcm*I at two sites in the polylinker produces T overhangs at the 3′ ends of the vector (Figure 3), which allows the cloning of Taq-generated PCR products (Testori *et al*., 1994).

Importantly, the pKAM554 plasmid and its derivatives contained a significantly shorter P_{GAPDH} fragment than that in pKAM444. The ability of the shortened promoter to ensure *APH* expression in *S. cerevisiae* at a level sufficient for

DOI: 10.1002/yea

primary selection of transformants was confirmed (data not shown).

Concluding remarks

In this study a selectable marker that allows primary selection of transformants in *E. coli* and yeast was developed and used to construct the pKAM444, pKAM554, pKAM555, pKAM556, pKAM567A and pKAM567B vectors (Figures 2, 3) bearing different sets of cloning sites. The specially designed *Xcm*I sites (Testori *et al*., 1994) in the pKAM567A and pKAM567B vectors allow their use for cloning of PCR products with A overhangs at 3′ ends. In contrast to the other vectors, pKAM556 contains a *HARS* for plasmid maintenance in *H. polymorpha* in the episomal state. The pKAM444 and pKAM554 plasmids contain only three cloning sites (*Cfr*I, *Pvu*II and *Pci*I) that can be useful when an excess of restriction sites is undesirable, e.g. for construction of plasmids destined for the integration into a host genome after linearization within the insert.

Importantly, the *HpMAL1* disruption cassette was constructed by the RAF-based approach, presuming integration of the entire plasmid into the target locus via double crossing-over (Figure 2). This approach has certain advantages over the classical approach, since it includes only one cloning step and allows the use of a single selectable marker for plasmid maintenance in *E. coli* and gene replacement in yeast.

In terms of convenience, RAF-based construction of cassettes for gene replacement can compete with purely PCR-based methods (reviewed by Wendland, 2003) if long flanking homology sequences are required for gene disruption (Wach, 1996; Gonzalez *et al*., 1999). Like the PCR-based methods, the RAF approach does not depend on restriction sites within the target gene. This is useful for the construction of precise junctions of the flanking homology sequences with the internal part of a disruption cassette. Moreover, this method allows replacement of a target locus with a long heterologous sequence (e.g. a large selectable marker or an expression cassette), which is difficult to amplify by PCR. Described plasmids are available from M. Agaphonov (moagaphonov@cardio.ru) for research purposes upon request.

Acknowledgement

This work was supported by Grant No. 09-04-01261 of the Russian Foundation for Basic Research.

References

Bogdanova AI, Agaphonov MO, Ter-Avanesyan MD. 1995. Plasmid reorganization during integrative transformation in *Hansenula polymorpha*. *Yeast* **11**: 343–353.

Chechenova MB, Romanova NV, Deev AV, *et al*. 2004. C-terminal truncation of α-COP affects functioning of secretory organelles and calcium homeostasis in *Hansenula polymorpha*. *Eukaryot Cell* **3**: 52–60.

Cregg JM, Barringer KJ, Hessler AY, Madden KR. 1985. *Pichia pastoris* as a host system for transformations. *Mol Cell Biol* **5**: 3376–3385.

Cregg JM, Russell KA. 1998. Transformation. In *Pichia Protocols*, Higgins DR, Cregg JM (eds). Humana: Totowa, NJ; 27–39.

Davies J, Jimenez A. 1980. A new selective agent for eukaryotic cloning vectors. *Am J Trop Med Hyg* **29**: 1089–1092.

Gatignol A, Baron M, Tiraby G. 1987. Phleomycin resistance encoded by the ble gene from transposon Tn 5 as a dominant selectable marker in *Saccharomyces cerevisiae*. *Mol Gen Genet* **207**: 342–348.

Gonzalez C, Perdomo G, Tejera P, *et al*. 1999. One-step, PCR-mediated, gene disruption in the yeast *Hansenula polymorpha*. *Yeast* **15**: 1323–1329.

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.

Inoue H, Nojima H, Okayama H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23–28.

Ito H, Fukuda Y, Murata K, Kimura A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**: 163–168.

Liiv L, Parn P, Alamae T. 2001. Cloning of maltase gene from a methylotrophic yeast, *Hansenula polymorpha*. *Gene* **265**: 77–85.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Sohn JH, Choi ES, Kang HA, *et al*. 1999. A dominant selection system designed for copy number-controlled gene integration in *Hansenula polymorpha* DL-1. *Appl Microbiol Biotechnol* **51**: 800–807.

Sohn JH, Choi ES, Kim CH, *et al*. 1996. A novel autonomously replicating sequence (ARS) for multiple integration in the yeast *Hansenula polymorpha* DL-1. *J Bacteriol* **178**: 4420–4428.

Ter-Avanesyan MD, Dagkesamanskaya AR, Kushnirov VV, Smirnov VN. 1994. The *SUP35* omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi$^+$] in the yeast *Saccharomyces cerevisiae*. *Genetics* **137**: 671–676.

Testori A, Listowsky I, Sollitti P. 1994. Direct cloning of unmodified PCR products by exploiting an engineered restriction site. *Gene* **143**: 151–152.

Wach A. 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**: 259–265.

Wendland J. 2003. PCR-based methods facilitate targeted gene manipulations and cloning procedures. *Curr Genet* **44**: 115–123.

Woodcock DM, Crowther PJ, Doherty J, *et al*. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res* **17**: 3469–3478.