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EFFECT OF THE KANAMYCIN RESISTANCE MARKER ON STABILITY OF 2m-BASED EXPRESSION PLASMIDS

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Abstract – In this paper we describe the effect of the kanamycin resistance gene (Km^r) on 2µm-based plasmid maintenance in *Saccharomyces cerevisiae*. The influence of this marker gene on the loss of the stable model-vectors proved to be constant, as well as independent of carbon source and culture growth rates. In strains for GAL_{UAS} – driven heterologous protein production introduction of Km^r resulted in curing of the yeast episomal plasmid (YEp) from the population in a small number of generations. Application of selective pressure on the strain producing recombinant penicillin G amidase (rPGA) did not provide the expected increase of protein yield. The influence of genetic elements for heterologous protein production on vector stability was examined, and the most destabilizing factors prove to be the presence and expression of the foreign gene.

Key words: 2µm plasmid, kanamycin resistance, penicillin G amidase, plasmid stability, *Saccharomyces cerevisiae*, selective markers.

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579.25

INTRODUCTION

Expression vectors based on the naturally occurring 2µm plasmid are the sole vectors in baker's yeast which satisfy two major biotechnological requirements: mitotic stability and high copy number. Vectors that employ the whole 2µm sequence have proved to be the most stable YEps (**y**east **e**pisomal **p**lasmid), since they contain all the sequences that control plasmid DNA replication and partitioning. However, artificially constructed 2µm-based vectors appear to be less stable than native 2µm plasmids, and are maintained at a lower copy number (Futcher and Cox, 1984). Alternative methods in yeast expression vector design include disintegrative vectors, which are recombination cointegrants made up of a 2µm-based vector and pRL (Chinerry and Hinchliffe, 1989). In the yeast cell, the cointegrant is resolved by (recombinase)-mediated *FRT* recombination into its two components. An *Escherichia coli* plasmid carrying the yeast *LEU2* marker and a single *FRT* (**F**LP **r**ecognition **t**arget) sequence (Bruschi and Howe, 1988), pRL is subsequently lost from the population because it is unable to propagate in *S. cerevisiae*. Disintegrative 2µm-based expression vectors were successfully applied in our laboratory for expression of the recombinant penicillin G amidase (penicillin acylase, PGA, *pac*) gene from the bacterium *Providencia rettgeri* (Ljubijankić et al., 1999; Ljubijankić et al., 2002) and gene for human interferon β (Todorović et al., 2000) in baker's yeast.

During our work with various Yeps, we realized the necessity for quick and exact monitoring of the mitotic stability of plasmids. For vectors whose presence is difficult or expensive to detect via a recombinant product, the introduction of a dominant selective marker is a logical alternative. Despite much effort to introduce novel marker genes (as listed in van den Berg and Steensma, 1997) into yeast biotechnology, there are few real dominant selective markers in *S. cerevisiae,* and their application is restricted. Although the majority of selective markers cannot be applied for large-scale production, their role in construction and selection of the optimal productive clones is irreplaceable. In order to retain a high yield of biomass, we decided to introduce the dominant selective marker gene for aminoglycoside

phosphotransferase I (APT I, *Km^r*), which confers resistance to kanamycin in prokaryotes and G418 in eukaryotes (Jimenez and Davies, 1980). The KanMX4 cassette (Wach et al., 1996) that we used has a dual (bacterial/fungal) promoter. The strong constitutive *TEF* promoter from the fungus *Ashbya gossypii* allows effective expression of the *Km^r* gene in yeast. A single copy of *Km^r* in *A. gossypii* confers resistance for up to 8 mg/ml of geneticin (Steiner and Philippsen, 1994).

However introducing a new gene could burden plasmid expression and plasmid mitotic stability, consequently decreasing the yield of the recombinant product. In order to examine the effect(s) of the dominant selective marker *Km^r* on vector stability, various *E.coli/S. cerevisiae* shuttle vectors were constructed based on disintegrative plasmids pBLU-D (Ludwig and Bruschi, 1991) and pGoB-2 (Ljubijankić et al., 1999). Plasmid pBLUR-D, with the yeast auxotrophic *URA3* marker gene, is the resolved form of pBLU-D (Fig. 1) and appears to be maintained at a high copy number without selective pressure. Construct pGoB-2 (Fig. 2) is the construct for *pac* expression in which maximal recombinant enzyme yield is provided by the BLITZ expression cassette (Ludwig et al., 1993). Expression from the BLITZ cassette is driven by the strong and strictly regulated *GAL1-10* promoter, which is repressed in the presence of glucose and induced about 1000-fold by galactose (Romanos et al., 1992).

The aim of this study was to test the dominant marker gene for G418 resistance as a useful tool for monitoring the proportion of plasmid-bearing cells in culture, while simultaneously examining the influence of the selective marker on YEp stability.

MATERIALS AND METHODS

Bacterial and yeast strains

The *E. coli* strain DH5α (*F^- Dlac U169 (F80 lacZ DM15) supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1*) (Hanahan 1983) was used to clone all the plasmids and constructs described in this work. The *S. cerevisiae* strain CBL1-30 (*MATα [cir^o] pep4*-3 *his3*Δ::*GAL10p-GAL4-URA3 leu2*-3,112 *trp1*-289 *ura3*-52 *can^R*), harboring plasmids pGoBR-2KS and pGIFNR (Todorović et al., 2000), has been described previously (Ludwig 1991). Isogenic ura$^-$ strain GSP-3 (*MATα [cir^o] pep4*-3 *his3*Δ::*GAL10p-GAL4-ura3*Δ247 *leu2*-3,112 *trp1*-289 *ura3*-52 *can^R*) was constructed as described previously

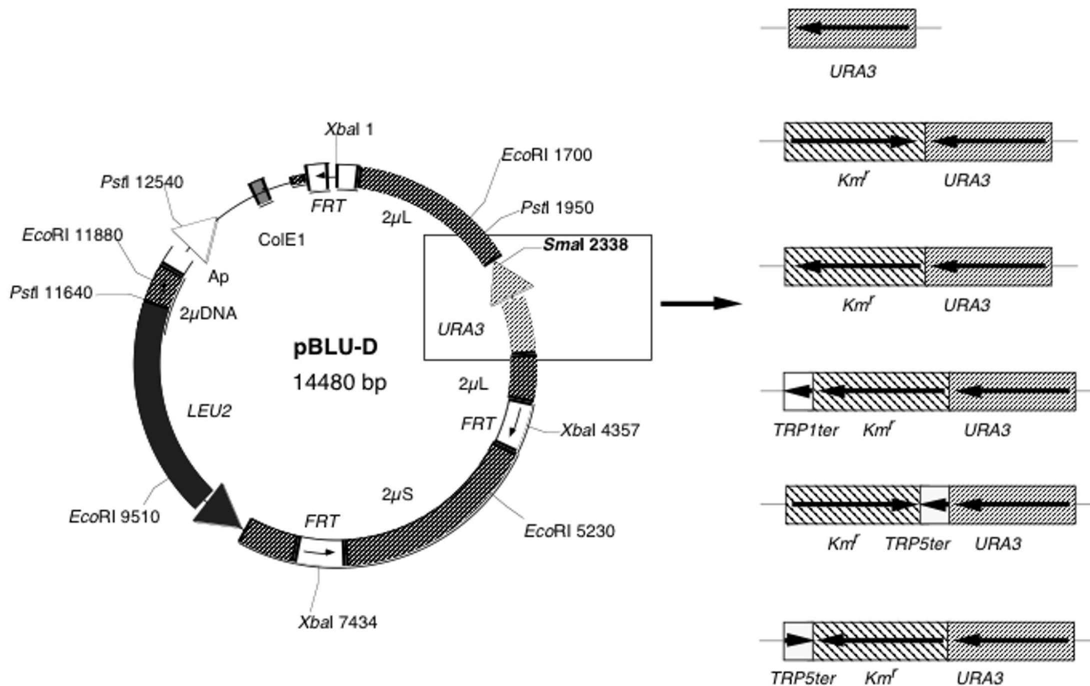


Fig. 1 - pBLU-based disintegrative vectors. The *Km^r* gene was cloned in the *Sma*I site of pBLU-D with or without different *TRP_{ter}* sequences in different orientations. The ure of pBLU-D was provided by courtesy of C. V. Bruschi.

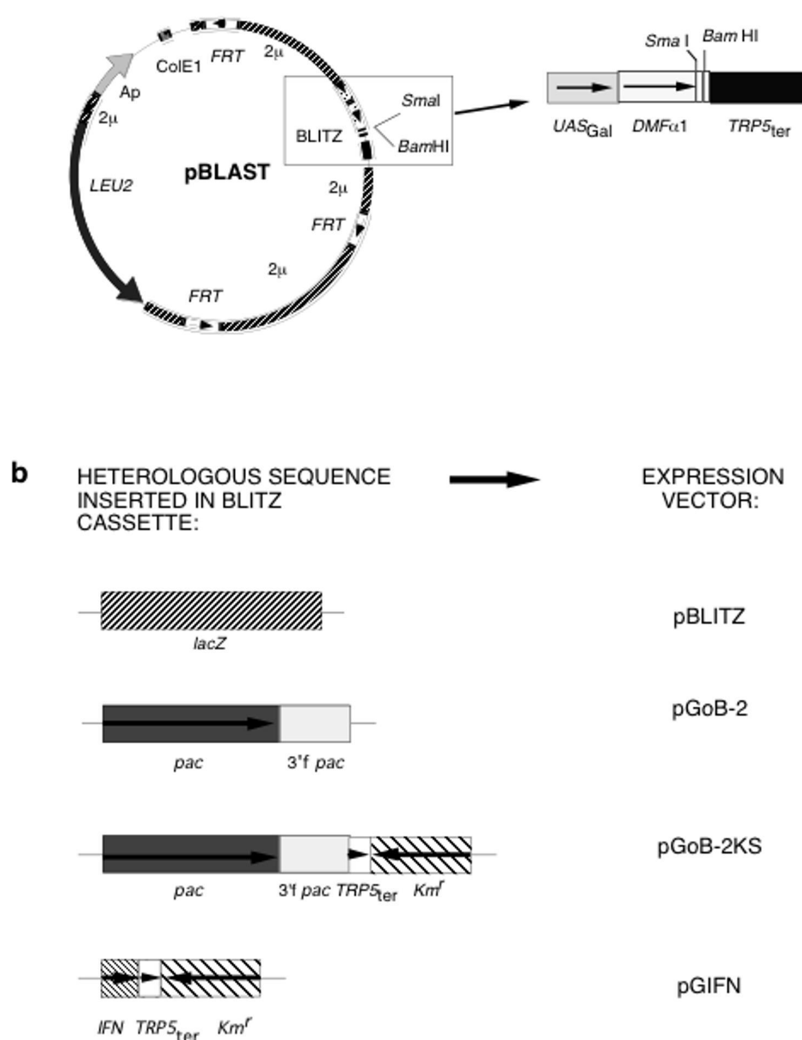


Fig. 2 - 2μm based vectors with the BLITZ expression cassette: a - Vector pBLAST gained after removing the *lacZ* reporter gene from pBLITZ contains the "empty" BLITZ expression cassette with *Sma*I and *Bam*HI sites for foreign gene insertion (Ludwig et al.*,* 1993). b - Different sequences inserted into the expression cassette resulted in different expression vectors. The 3'f *pa - pac* gene was cloned together with the downstream fragment of the *P. rettgeri* chromosome. The figure of pBLAST was provided by courtesy of C. V. Bruschi.

(Pavković & Ljubijankić, 2000) in order to provide an appropriate genetic background for testing the plasmid stability of *URA3* carrying pBLU-based vectors. Briefly, the *URA3* gene on the CBL1-30 chromosome was replaced with the inactive allele *ura3*Δ247, which was obtained by deletion of the 247 bp *Stu*I/*Eco*RV fragment of the *URA3* gene on the YIplac211 plasmid (Gietz and Sugino, 1988). The inactive allele was introduced into competent CBL1-30 yeast cells by electroporation. The Ura^- transformants were scored on $DOura^-$ medium with 5-fluoroorotic acid (Boeke et al.*,* 1984).

Media and culture conditions

Esherichia coli strains were grown in LB medium with or without agar (20 *g*/L). Ampicillin was added at 100 $\mu g/\mu l$ and kanamycin at 60 $\mu g/\mu l$ for selection of recombinant plasmids. *Saccharomyces cerevisiae* strains were grown in one of the following standard yeast media: semidefined rich YPD (1% yeast extract, 2% peptone, 2% glucose), YPRaf (1% yeast extract, 2% peptone, 2% raffinose), and YPGal (1% yeast extract, 2% peptone, 2% galactose), with or without addition of 200 $\mu g/\mu l$ of G418 (geneticin, G418 sulfate Gibco BRL, Gaithersburg, MD);

and the defined yeast synthetic complete media, leucine-less ($DOleu^-$) and uracil-less ($DOura^-$), described by S h e r m a n et al. (1986). For *pac* expression, a self-inductive medium was used (YPGal with addition of 0.2% glucose).

DNA transformation and recombinant DNA techniques

The DH5α *E. coli* strain was transformed as described by I n o u e et al. (1990). Yeast transformation by electroporation was performed according to M e i l h o c et al. (1990). The transformation efficiency of *S. cerevisiae* was in the order of magnitude of 10^3 transformants/mg of DNA. *Escherichi coli* plasmid DNA was isolated using the QIAGEN Plasmid Kit and the QIAGEN Mini-prep Kit (QIAGEN) or as described by D e l S a l et al. (1989). Manipulations of DNA such as restriction enzyme digestions, ligations and agarose gel

Table 1. List of plasmids used in this work. [a] See text for details.

E. coli vectors		
Plasmid	Relevant characteristic[a]	Source or reference:
pFAKanMX4	Ap^r, Km^r	W a c h et al., 1996
pUC18/19	Ap^r	Y a n i s c h - P e r r o n et al., 1985
pUC19T	Ap^r, *S. c.* $TRP1_{ter}$	S t o r i c i , F.
pUC18TK	Ap^r, *S. c.* $TRP1_{ter}$, Km^r	this paper
pUC19TK	Ap^r, *S. c.* $TRP1_{ter}$, Km^r	this paper
pUC18KT5	Ap^r, *S. c.* $TRP5_{ter}$, Km^r	this paper
pUC19KT5	Ap^r, *S. c.* $TRP5_{ter}$, Km^r	this paper
E. coli / S. cerevisiae shuttle vectors		
Plasmid	Relevant characteristic[a]	Source or reference:
pBLU-D	pRL + URA3	Ludwig and Bruschi, 1991
pBLU-KP	pRL + URA3, Km^r	this paper
pBLU-KS	pRL + URA3, Km^r	this paper
pBLU-KT5P	pRL + URA3, Km^r, $TRP5_{ter}$	this paper
pBLU-KT5S	pRL + URA3, Km^r, $TRP5_{ter}$	this paper
pBLU-TK	pRL + URA3, Km^r, $TRP1_{ter}$	this paper
YCpKan	pRL + Ap^r, Km^r, BLITZ cassette, $TRP5_{ter}$	T o d o r o v i ć et al., 2000
pGIFN	pRL + Km^r, BLITZ cassette, hIFN-β	T o d o r o v i ć et al., 2000
pGoB-2	pRL + BLITZ cassette, *P. r. pac*	L j u b i j a n k i ć et al., 1999
pGoB-2KS	pRL + Km^r, BLITZ cassette, *P. r. pac*	T o d o r o v i ć et al., 2000

electrophoresis were performed as described by Maniatis et al. (1982).

DNA hybridization

Total yeast DNA was isolated according to Ausubel et al. (1989), digested with *Eco*RI, transferred from an agarose gel onto a nylon membrane, and hybridized by the Southern procedure (Maniatis et al., 1982). Plasmid DNA was detected with a probe comprising the 428 bp *Hin*dIII/*Nco*I fragment of the Km^r gene. The probe for genomic DNA was synthesized from *S. cerevisiae* gene *VMA22* (generated by a PCR with primers 5'-AGGATCCGGCTTAACGAGAAG-3' and 5'-AGGATCCTTTTTCAAACCATGGAC-3').

We decided to use the *VMA22* gene probe as a control because it is present in one copy per haploid yeast genome (Skoko et al., 2005) and primers were already available. The DNA probes were labeled with [α-^{32}P]dCTP by random primer extension (Multiprime DNA labeling system, Amersham). Hybridization was detected by autoradiography, and the plasmid copy number was determined densitometrically by comparing the signal of the single-copy gene to the plasmid DNA signal in the same lane (BioDocAnalyze 1.0, Biometra, Göttingen, Germany).

Plasmid construction

A list of plasmids used in this study is given in Table 1. The plasmid pBLU-D was used for all subsequent constructions described in this paper. Construction of the plasmids YCpKan, pGoB-2KS, and pGIFN was described previously (Todorović et al., 2000).

1. Plasmids pBLU-KP and pBLU-KS: the1483 bp *Eco*RV/*Sma*I fragment with the KanMX4 cassette from pFAKanMX4 was inserted in both orientations into the pBLU-D vector digested with *Sma*I.

2. Plasmid pBLU-TK: the Km^r gene obtained as a *Sca*I/*Sma*I fragment from pFAKanMX4 was ligated with the pUC19T vector linearized with *Sma*I to give pUC19TK. The Km^rTRP1_{ter} cassette obtained by digestion of pUC19TK with *Sph*I and *Nde*I was inserted into pUC18 digested with the corresponding enzymes, after which pUC18TK was then digested with *Sma*I and the obtained 1624 bp Km^rTRP1_{ter} cassette was ligated into pBLU-D previously linearized with *Sma*I.

3. Plasmids pBLU-KT5P and pBLU-KT5S: the 2681 bp *Bgl*II/*Sal*I Km^rTRP5_{ter} cassette from YCpKan was first inserted into pUC19 digested with *Bam*HI and *Sal*I restriction enzymes and then recovered as a 1827 bp fragment by digestion with *Sma*I and ligated in both orientations to pBLU-D previously linearized with *Sma*I.

Resolution of vectors after transformation

A GSP-3 strain was transformed with pBLU-based vectors. Immediately after transformation, the cells were plated onto DOleu$^-$ plates. When colonies appeared, at least three individual transformants of each plasmid type were picked and cultivated for 30 generations in YPD medium in order to allow full resolution of disintegrative plasmids and loss of pRL from the population. Cultures were then diluted and plated onto YPD plates to yield ~200 colonies per plate. Colonies were subseqently tested for the loss of the carrier plasmid pRL by replica-plating (as mini streaks) onto DOleu$^-$ minimal medium, and for the presence of resolved expression plasmids pBLUR-KP, pBLUR-KS, pBLUR-TK, pBLUR-KT5P, and pBLUR-KT5S by replica plating onto YPD_{G418} selective plates.

Determination of 2ìm-based plasmid stability

Plasmid stability was determined according to the modified method of Mann and Davies (Gietz & Sugino, 1988) by cultivating on selective and nonselective media. Testing in non-selective conditions was expanded to a nonselective repressive medium with glucose (YPD) and a nonselective inductive medium with galactose (YPGal). Cells were cultivated for 12 generations in $YPRaf_{G418}$ – a selective medium with a neutral carbon source – to ensure plasmid presence in the population and to avoid the effects of glucose and galactose on $GAL1\text{-}10_{UAS}$ driven expression. A portion of the culture was diluted and plated onto YPD plates to give 200-300 colonies per plate for monitoring of plasmid presence in the starter culture. Inocula (0.02%) of the starter culture ($3x10^5$ cell/ml) were transferred to parallel YPD and YPGal cultures and cultivated for 36 generations. On the basis of our previous work in establishing optimal culture conditions for *pac* expression (Ljubijankić et al., 2002), we concluded that a 36-generation cultivation is sufficient for plasmid instability. To maintain cultures in log phase, 0.02% inocula of the cultures were transferred to fresh media (batches I, II, and III) every 12 generations. To monitor plasmid stability, a part of the cultures from the first and the third batch were diluted and plated

onto YPD plates to yield 200-300 colonies per plate. Colonies from YPD plates were then replica-plated onto selective YPD_{G418} plates and the number of colonies grown on the YPD plates (100%) was compared to the number of colonies grown on the selective plates. Plasmid stability was calculated from the formula given by C h r i s t i a n s o n et al. (1992):

$$PLR = 1 - e^{\frac{\ln(P2/P1)}{g}} \text{ [plasmid/(cell)(division)]}$$

PLR is the plasmid loss rate; P1 is the number of colonies grown on the nonselective plate; P2 is the number of colonies grown on the selective plate; and g is the number of generations.

The determined plasmid loss rate expressed as the number of plasmid-free segregants in the population after a single division is the mean of at least two independent experiments. Cultures were subsequently pelleted and total yeast DNA was isolated for estimation of the plasmid copy number.

pac expression and assay of PGA activity

A mesured volume (10 ml) of self-inductive medium was inoculated from the first overnight $YPRaf_{G418}$ cultures of *pac* carrying clones (pGoBR-2/CBL1-30, pGoBR-2KS/CBL1-30). After 84 h of growth, 1 ml was subtracted to measure rPGA activity using a standard colorimetric method (A l k e m a et al., 1999). One unit of enzyme activity was defined as the quantity of PGA catalyzing hydrolysis of 1 µmol of 6-nitro-3(phenylacetamido) benzoic acid per minute at 25ºC.

RESULTS

Growth rates

Beyond the inducing effect on *GAL* promoters, galactose also has a general effect on the growth rate of yeast cultures. Growth rates for CBL1-30 and GSP-3 strains in rich media with different carbon sources (2% raffinose, 2% glucose, and 2% galactose) were measured (data not shown). We confirmed that the isogenic strains CBL1-30 and GSP-3 had the same generation time, approximately 2 h in glucose and raffinose, and approximately 8 h in galactose (data not shown). Plasmid stability experiments were therefore performed in the same conditions regarding the carbon source: the starter culture had an optimal growth rate in a neutral carbon source, and the plasmid stability assay was performed in the media with optimal (glucose) and slow (galactose) growth rates for all vectors with or without GAL_{UAS}.

Resolution of vectors after transformation

In the case of pBLU-based plasmids, after thirty generations of nonselective growth less than 20% of the cells contained both selective markers (Leu^+G418^+ phenotype), while the remaining cells contained only the Km^r marker (Leu^-G418^+ phenotype). Colonies with the Leu^-G418^+ phenotype were chosen to determine the stability of 2µm-based plasmids alone. For this reason from this point the letter "R" is added to plasmid names referring to the resolved plasmid form.

The stability of pGoB-based vectors

Stability of pGoBR-2 was estimated during rPGA production (L j u b i j a n k i ć et al. 2002), and the number of plasmid-bearing cells was estimated via the number of PGA^+ clones. Comparison of the stabilities between the parental pGoBR-2 and plasmid pGoBR-2KS showed that in noninductive conditions pGoBR-2 was completely stable (99% of PGA^+ clones), while pGoBR-2KS had a plasmid loss rate of 5.15% per generation. Estimated plasmid loss for pGoBR-2 was 15% per generation in the first 24 h after galactose induction (L j u b i j a n k i ć, unpublished results). As for pGoBR-2KS, it

Table 2. Loss rates of pGoB-based expression vectors expressed as % of plasmid free cells in population after one division.
PLR - plasmid loss rate, calculated as described in Materials and Methods.

plasmid	media	PLR/generation (%)
pGoBR-2KS	YPD	5.15±0.41
	YPGal	14.02
pGIFNR	YPD	2.46±0.32
	YPGal	7.60±2.40

showed a similar loss rate of approximately 15% per generation when cultivated in galactose, white pGIFNR exhibited plasmid loss rates of 2.46% on glucose and 7.60% on galactose and appeared to be twice as stable as isogenic pGoBR-2KS (Table 2).

Comparison of rPGA production

Clones carrying vectors with the BLITZ expression cassette for the production of recombinant PGA were cultivated and induced for foreign protein production as described in Materials and Methods. In self-inductive medium after 12 h of cultivation, yeast cells exhaust all the glucose and switch to utilization of galactose, leading to *pac* induction. Activities of PGA after 72 h of induction are shown in Fig. 3a. Even after optimization of culture growth conditions and constant selective pressure, the production of rPGA by the pGoBR-2KS clone was barely one third of the production by the clone carrying pGoBR-2. The low activity of the pGoBR-2KS clone in producing rPGA is probably due to its diminished stability in glucose, resulting in a small number of cells in the population containing the *pac* gene at the beginning of induction. Additionally, the production of rPGA per cell did not differ considerably between pGoBR-2KS and pGoBR-2 clones (Fig. 3b).

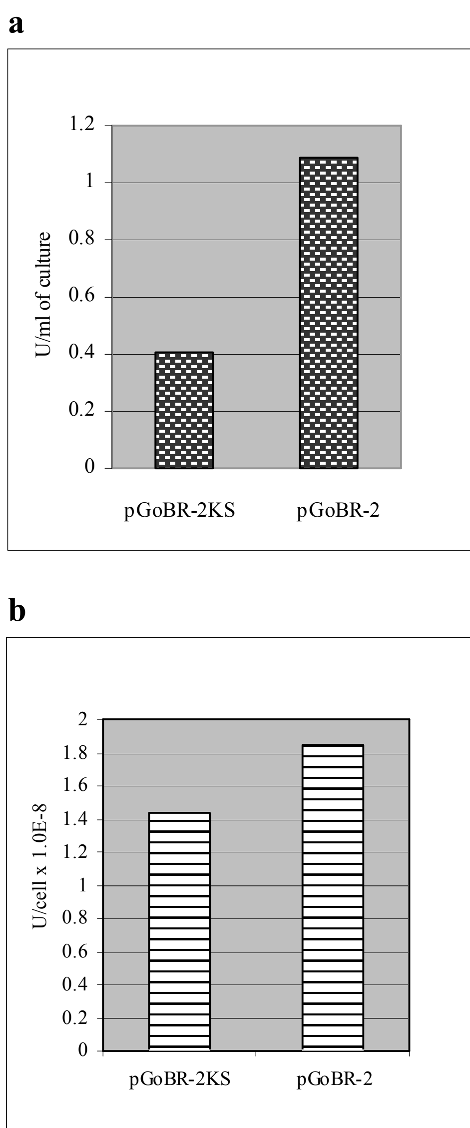


Fig. 3 - Comparison of PGA activities between clones carrying pGoBR-2KS and pGoBR-2. 3a - Overall production of rPGA in CBL1-30/pGoBR-2KS and CBL1-30/pGoBR-2 clone 72 h after the induction. 3b - Production of rPGA per PGA^{+} cell.

Stability of pBLU-based vectors

To define the direct contribution of the *Km^r* gene to plasmid stability, pBLU-KP and pBLU-KS were constructed and introduced into yeast cells, where they resolved into pBLUR-KP and pBLUR-KS. The *Km^r* gene orientation had no impact on their stability in glucose medium, since the plasmid loss rates of these vectors were identical. When cultivated for 36 generations on galactose, plasmid loss rates were 0.07% for pBLUR-KP and 0.93% for pBLUR-KS, suggesting that prolonged generation time does not contribute to plasmid instability either. It should be pointed out that plasmid pBLUR-KP exhibited considerable discrepancy of stability while cultivated in galactose medium, both within a single experiment and between independent experiments. Plasmid loss rates are given in Table 3.

In pGoB-2KS and pGIFN constructs, the KanMX4 cassette was cloned together with the inverted TRP5 terminator sequence from transient vector YcpKan (Todorović et al., 2000). Inverted $TRP5_{ter}$ was regarded as not important for heterologous gene expression. The pBLU-KTP and pBLU-KTS constructs were created in order to test the possible influence of inverted *$TRP5_{ter}$* and the KanMX4 cassette on plasmid stability, since such an assembly of genetic elements exists in the most unstable pGoB-based vectors. The stability of pBLUR-KTP/S, both on glucose and galactose, did not differ from that of the other *Km^r* constructs.

Efficient termination of foreign gene transcription is one of the factors that generally influence gene expression in yeast (Bijvoet et al., 1991). Because the BLITZ cassette contains a promotor and a terminator of different strengths, we tested the possibility that inefficient termination could contribute to plasmid instability by carrying on transcription through the *STB* locus. The *$GAL1\text{-}10_{UAS}$* sequence is one of the strongest yeast acti-

Table 3. Loss rates of pBLU-based model vectors expressed as % of plasmid free cells in population after one division.
PLR - plasmid loss rate, calculated as described in Materials and Methods.

plasmid	media	PLR/generation (%)
pBLUR-D	YPD	0
	YPGal	0.07
pBLUR-KP	YPD	0.53 ± 0.05
	YPGal	0.07 ± 0.32
pBLUR-KS	YPD	0.53 ± 0.05
	YPGal	0.93 ± 0.03
pBLUR-TK	YPD	0.32 ± 0.05
	YPGal	0.36 ± 0.17
pBLUR-KT5P	YPD	0.66 ± 0.13
	YPGal	0.64 ± 0.14
pBLUR-KT5S	YPD	0.61 ± 0.05
	YPGal	0.72 ± 0.67

vating sequences, but the *TRP5* terminator corresponds to a *TRP5* promoter of medium strength. We replaced TEF_{ter} from *A. gossypii* in the KanMX4 cassette (S t a i n e r & P h i l i p p s e n, 1994) with $TRP1_{ter}$ from *S. cerevisiae* to mimic conditions of intensive transcription of a foreign gene with a promoter and terminator of different origin and strength. The plasmid pBLU-TK was constructed and its stability tested. Plasmid loss rates were the same as in constructs carrying the Km^r gene with TEF_{ter}.

Copy number

The plasmid copy number (PCN) changed in the population during nonselective growth, as did the percentage of plasmid-carrying cells. Since the number of plasmid-carrying cells declined after every doubling, thereby reducing the number of cells able to segregate in future divisions, PCN was normalized for plasmid loss rates (Table 4). All pBLU-based plasmids showed identical and stable PCN values which were considerably reduced once selective pressure had been removed. In pGoB-based plasmids, PCN was irregular and varied (Fig. 4). Cells cultivated in glucose had a lower, yet more stable PCN. The pGIFNR plasmid appeared to be present in a considerably lower copy number than the isogenic pGoBR-2KS.

DISCUSSION

Production of heterologous proteins in baker's yeast is influenced by many factors (for review see R o m a n o s et al., 1992). Gene dosage is considered to be one of the most important. When expression is from YEp vectors, gene dosage depends on plasmid stability in the population and on plasmid copy number.

Cultivation on a neutral carbon source in the presence of G418 showed that the population of pBLU-based clones and pGIFNR contained 1-10% of plasmid-free segregants. Monitoring of plasmids with auxotrophic selective markers (G i e t z & S u g i n o, 1988; C h r i s t i a n s o n et al., 1992) revealed that even under selection, plasmid-free cells encompassed 5-30% of the population. This represented the balance between generation of new and loss of old plasmid-free segregants. However, the pGoBR-2KS clone contained 40% of plasmid free segregants after 10 generations. Plasmids that are pGoB-based usually have high average copy numbers in the cell, but clonal variation may lead to differences in stability between clones of the same construct. Presumably, those clones with low PCN lose plasmids rapidly, since plasmids that have lowered PCN also tend to show segregational instability (F u t c h e r & C o x, 1984; and K i r k p e k a r & G u l l ø v, 1996). In the case of pGoBR-2KS, clonal variation seems to be significant.

In culture phase-induction experiments (L j u b i - j a n k i ć, unpublished results), the CBL1-30/pGoBR-2 culture induced by galactose in early growth stages contained a smaller number of plasmid-bearing cells. Most of the plasmid-bearing cells as well as the highest yield of recombinant PGA coincided with the minimal number of divisions that the culture underwent during the induction. The considerable difference in stability of pGoB plasmids cultivated on glucose vs. ones cultivated on galactose is not uncommon, since other 2μm-based expression vectors, as well as *ARS* vectors containing a galactose-dependent promoter, show reduced stability,

depending on the carbon source (Bitter & Egan, 1988; Kirkpekar & Gulløv, 1996).

Plasmid loss in CBL1-30/pGoBR-2KS and CBL1-30/pGIFNR clones during growth in glucose could be additionally attributed to constitutive expression of *Km*^{r} and overabundance of its product. The KanMX4 cassette is effectively expressed owing to its strong *TEF* promoter. A single copy of this cassette conferred resistance in *A. gossypii* to geneticin up to 8 mg/ml (Steiner & Philippsen, 1994). Efficiency of the *TEF* promoter in baker's yeast is enough to allow resistance to 200 µg/ml G418, even in the presence of a single copy [(as shown for the KanMX4 cassette used in ORF deletions and construction of ORF replacement cassettes in *Saecharomyces cerevisiae,* Wach et al., (1996)]. Therefore, cells could afford to lose most of their plasmid molecules and still remain G418-resistant. A considerable effect is also realised through accumulation of the *Km*^{r} gene product, APT, which remains in the cell after loss of the plasmid. Aminoglycoside phosphotransferase is stable in yeast cells, and its half-life was calculated to be about 7.5 h (Hadfield et al. 1990). Thus, cultures at the optimal growth rate should pass another two or three divisions before the steady-state level of residual APT is reduced by one half.

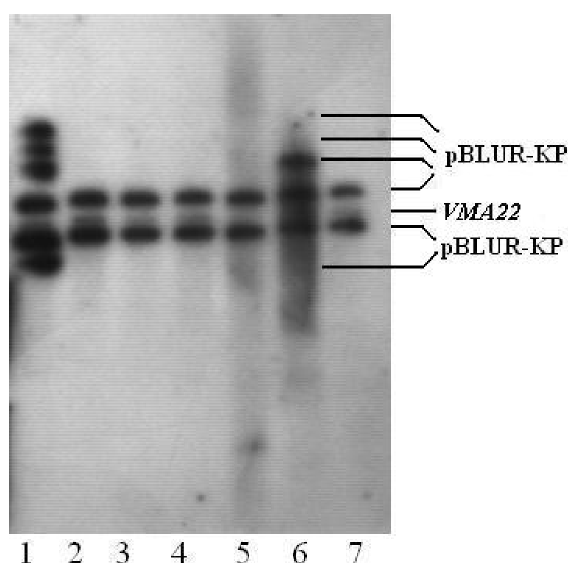
The low activity of the pGoBR-2KS clone in producing PGA is probably due to diminished stability on glucose and the small number of cells containing the *pac* gene in the population at the time of induction, since similar rPGA production per cell suggests an equal ability of the single clone to produce recombinant protein.

When recombinant protein is produced from a vector containing the BLITZ cassette, such as pGoB-based vectors, plasmid instability is also connected to the actual process of foreign gene expression. The cassette contains 365 bp of the intergenic region, *GAL1-GAL10* with a *UAS* element, the TATA box and leader sequence of MFα1, and the terminator region of the yeast *TRP5* gene (Ludwig et al., 1993). Neither of these genetic elements influences plasmid stability simply by its presence [*GAL1-10*$_{UAS}$ was cloned in different orientation on plasmids pBLU-G and pBLU-GC (not shown), *TRP5*$_{ter}$ on plasmids pBLU-KTP/S]. The *GAL1-10*$_{UAS}$ sequence is one of the strongest yeast-activating sequences, but the *TRP5* terminator corresponds to a *TRP5* promoter of medium strength. It was previously shown that a bidirectional promoter of medium capacity, *TRP1*$_{ter}$, was not sufficiently effective in termination of transcription from the *GAL1* gene (Irniger et al., 1991). We did not discern that discordance in strength and origin of the promoter and terminator played any role in plasmid instability (to judge from model plasmid pBLUR-TK). This suggests that, in the case of pGoBR-2KS and pGIFNR, the intensity of expression from foreign sequences is the main reason for plasmid instability. The transcriptional status and nature of both heterologous genes (gene of

Table 4. Estimation of plasmid copy number (PCN). pBLU - pBLU-based plasmids; I - PCN after 12 generations of growth; II - PCN after 24 generations of growth; III - PCN after 36 generations of growth. *Note*: Where two measurements were made, both values are listed, and the average value is shown in parentheses.

Medium	YPRaf$_{G418}$	YPD			YPGal		
batch	I	I	II	III	I	II	III
pBLU	50	15-20	15-20	15-20	15-20	15-20	15-20
pGIFNR	27 4 (15)	24 3 (14)	12 3 (7)	21 1 (11)	1 10 (6)	1 3 (2)	3 4 (4)
pGoBR-2KS	42	31	29	59	220	17	20

interest and Km^r marker) probably impaired the maintenance of expression vectors, although all vital sequences of 2µm remained intact. In a comparative study of four auxotrophic markers, U g o l i n i et al. (2002) demonstrated how the level of expression on a plasmid is inversely correlated with plasmid stability and copy number, and - hypothetically - in accordance with its strong secondary, hairpin like, DNA structure. The synergistic effect of strong expression from $GAL1\text{-}10_{UAS}$ and *TEF* promoters could impair replication and partition functions by disturbing plasmid secondary structure. Size of the *pac* gene apparently plays a role in the high instability of pGoBR-2KS, as it is the feature most divergent from its more stable twin plasmid, pGIFNR.



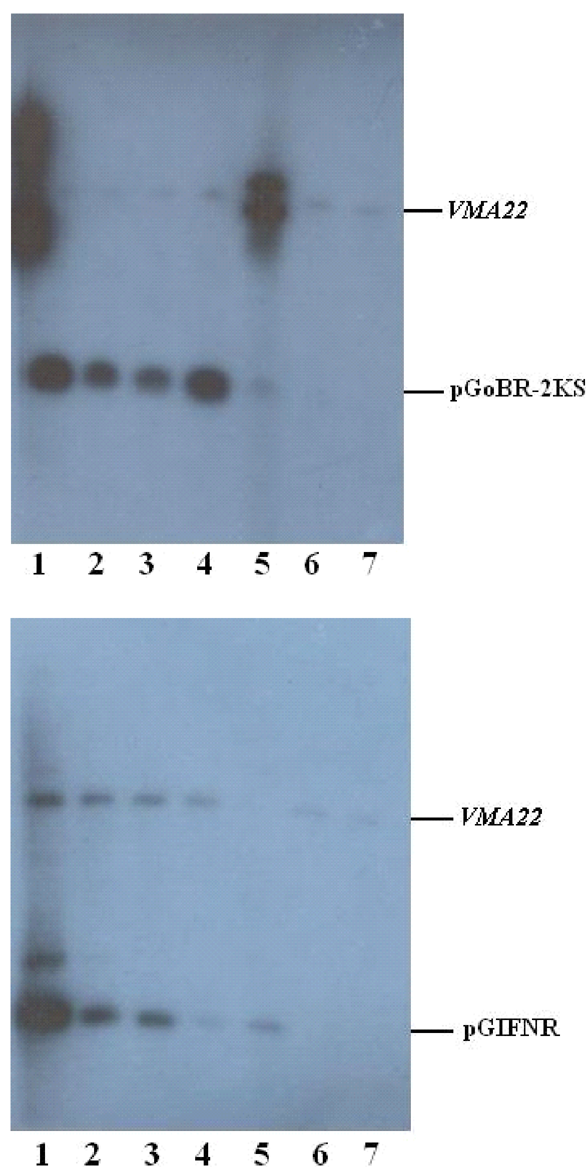


Fig. 4 – Southern blot analysis of total DNA from *Saecharomyces cerevisiae* clones carrying plasmids pBLUR-KP, pGIFNR, and pGoBR-2KS. Each lane contains a signal from the plasmid DNA and a signal from the chromosomal fragment with a single-copy *VMA22* gene. Lane 1: total yeast DNA isolated from the $YPRaf_{G418}$ culture; lanes 2-4: total yeast DNA isolated from the YPD culture (after 12, 24, and 36 generations of growth, respectively); lanes 5-7: total yeast DNA isolated from the YPGal culture (after 12, 24, and 36 generations of growth, respectively). * - in lane 5, the signal from *VMA22* is partly masked by incompletely digested pGoBR-2KS.

The stability of pBLU-based plasmids was reduced =1% by expression of the gene for kanamycin resistance. In vectors for $rPGA_{P.\ rett.}$ expression, the effect of Km^r introduction was manifested as a decrease in the yield of enzyme, since recombinant product formation mirrored vector stability. The KanMX4 cassette conferring a high level of resistance proved to be an inadequate tool for monitoring baker's yeast clones carrying multicopy vectors for heterologous protein production. This should be taken into account as a general principle in the choice of *S. cerevisiae* selective markers. Ideally, resistance should be provided not by a high level of expression, but also through a large number of gene copies, as in the case of the auxotrophic markers *leu-2d* and *ura3-d* (B e g g s, 1978; L o i s o n et al., 1989).

Abbreviations: Ap – ampicillin; APT - aminoglycoside phosphotransferase; Flp - *S. cerevisiae* recombinase; *FRT* - Flp recognition target; Km - kanamycin; MFα1 - mating factor α;

pac – gene for penicillin G amidase; PGA – penicillin G amidase; *STB* - 2μ stabilization locus; TEF - translation elongation factor 1 alpha; *UAS* - upstream activating sequence; *VMA22* - gene for one of the subunits of yeast V-ATPase.

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УТИЦАЈ МАРКЕР ГЕНА ЗА КАНАМИЦИНСКУ РЕЗИСТЕНЦИЈУ НА СТАБИЛНОСТ 2m ЕКСПРЕСИОНИХ ПЛАЗМИДА

НАДА СТАНКОВИЋ, БРАНКА ВАСИЉЕВИЋ и †ГОРАН ЉУБИЈАНКИЋ

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У овом раду описан је утицај гена за канамицинску резистенцију (Km^r) на одржавање 2μm плазмида у *Saccharomyces cerevisiae*. Присуство овог маркер гена доводи до губитка стабилног модел-вектора константном стопом независном од извора угљеника и стопе раста културе. Код сојева за синтезу хетерологних протеина са галактозног промотора (GAL_{UAS}) увођење Km^r резултира „чишћењем“ од квашчевих епизомалних плазмида (YEp) у свега неколико генерација. Примена селективног притиска на сојеве који производе рекомбинантну пеницилин G амидазу (rPGA) није довела до очекиваног повећања приноса протеина. Испитивањем утицаја самих генетичких елемената за производњу хетерологних протеина на стабилност вектора показано је да најјаче дестабилишуће дејство има присуство и експресија страног гена.