Effect of the defective leucine gene *leu2-d*, on the properties of recombinant plasmid in yeast *Saccharomyces cerevisiae*

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The presence of a defective leucine gene *leu2-d* ensures a high stability of recombinant plasmid pYEXBK (95–98%) under both non-selective and selective conditions in yeast strains marked by *ura3-52 leu2* mutations. In *ura3-52* strains, the effect of *leu2-d* (pYEXBK) or its inactivation (pYEXBK-Δleu) does not influence the stability of plasmid. The simultaneous action of the K2 killer preprotoxin and defective leucine gene leads to formation of an additional group of transformants with altered immunity ($K2^+R1^+R2^+R28^{\pm}$). It was determined that expression of K2 killer toxin increases 1.5–2 times under the action of the *leu2-d* marker gene, indicating an increased plasmid copy number.

Key words: *Saccharomyces cerevisiae*, *leu2-d*, K2 killer preprotoxin gene

INTRODUCTION

Usually, selection of transformants in yeast *Saccharomyces cerevisiae* is based on the presence of selectable markers. The markers *LEU2* and *URA3* are among those most widely used. They function as a dominant selectable marker only when the recipient yeast cell has a recessive mutation in the corresponding chromosomal copy of the cloned gene. The *LEU2* gene encodes β-isopropylmalate (β-IPM) dehydrogenase, which catalyzes the third step in leucine biosynthesys [1]. Unlike several other yeast genes involved in amino acid biosynthesis, *LEU2* is under specific amino acid control: the gene expression is repressed by elevated concentrations of leucine [2]. The gene for *leu2-d* is a poorly expressed allele of *LEU2* bearing thymine in 863 position instead of cytosine, althought without altering the codon sense [3]. Also, *leu2-d* has a deletion of the 5'-flanking region of the *LEU2* message which leaves only 29 bp preceeding the *LEU2* initiation codon [2]. This derivative of the *LEU2* gene (when present on a YEp plasmid) requires a very high plasmid copy number to give a Leu^+ phenotype [2] and thereby increase the stability of 2 μm plasmid derivatives under non-selective conditions [3]. Under leucine deprivation the copy number of *leu2-d* plasmids rises sufficiently high to cure the endogenous 2-μm plasmid [4]. As *leu2-d* is a poor selectable marker for Li^+-mediated transformation, *URA3* is sometimes included [3]. The cloned *leu2-d* gene can also complement mutations in the *leuB6* gene of *E. coli* [5]. The availability of bacterial mutations that can be complemented by yeast genes can greatly simplify plasmid constructions allowing genetic screening for the expected recombinant plasmid. Analysis of the impact of *leu2-d* gene on the properties of the recombinant plasmid pYEXBK formed in yeast *S. cerevisiae* by recombination *in vivo* is presented in this study.

MATERIALS AND METHODS

The *S. cerevisiae* strain α'1 (*MATα leu2-2 [KIL-0]*) was used as a sensitive tester strain for killer toxin activity [6]. For testing the immunity we used four yeast strains: K7 (*MATa arg9 [KIL-K1]*), Rom K100 (*wt, HM/HM [KIL-K2]*), M437 (*wt, HM/HM [KIL-K2]*) and K28 (*wt, HM/HM [KIL-K28]*) [6]. Yeast strains 3PMR-1 (*MATα ura3-52 [KIL-0]*) and 21PMR (*MATα ura3-52 leu2 [KIL-0]*) [6] as well as H13 (*MATα ura3-52 leu2[Kil-0]*) and H14 (*MATa ura3-52[KIL-0]*) (the two latter – monospore segregants of hybrid DBY4947 x α'1) were used for investigation of gene expression from the recombinant plasmid. The *E. coli* strain DH5α was used for amplification of DNA plasmids [7].

Media for the growth of *S. cerevisiae* as well as standard genetic techniques have been described in Sherman et al. [8]. All media for routine growth and maintenance of the *E. coli* strain were standard [9].

Plasmid pYEX12 codes *LEU2* marker gene as well as K2 killer preprotoxin gene (under the control of ADH1 promoter) [10]. The recombinant plasmid pKV lacks usual selective markers and contains only a functional K2 preprotoxin gene [11]. Gene coding for urokinase, *URA3* and *leu2-d* genes were present in the multicopy plasmid pSU [3, 12].

General procedures for the construction and analysis of recombinant DNAs were performed as described by Sambrook et al. [9]. All restriction enzymes, T4 DNA ligase, bacterial alkaline phosphatase, Klenow fragment and DNA size markers were obtained from MBI Fermentas (Vilnius) and used according to the manufacturer's recommendations. Transformation of *S. cerevisiae* was performed by lithium chloride procedure of Ito et al. [13]. Transformants were selected by complementation of auxotrophic markers, clones were checked for toxin production by replica-plating to a lawn of *S. cerevisiae* strain α'1.

The ability of a strain to kill and immune was assayed on indicatory MB media essentially as described in [14, 15]. The stability of the Ura^+, Leu^+, $K2^+$ phenotype of transformants bearing the pYEXBK plasmid was analysed by growing cell colonies on non-selective (YEPD) medium for 3 days at 30 °C and plating onto selective media: minimal – in case of *leu2-d* and *URA3*; indicatory MB with a layer of α'1 – in case of K2 preprotoxin gene.

RESULTS AND DISCUSSION

Taking advance of interplasmid recombination *in vivo* and further autoselection of resulting constructs, the recombinant plasmid pYEXBK has been obtained. For this purpose, a pKV bearing K2 preprotoxine gene has been co-transformed with *URA3*, *leu2-d* and urokinase genes-containing plasmid pSU into the *S. cerevisiae* strain 3PMR-1*ura3-52*. Successful colonies were found to undergo genomic destabilization with the following plasmid–strain autoselection [16]. Extensive restriction analysis of the resulting plasmid confirms the presence of phenotypically determined yeast markers *URA3* and *leu2-d* as well as K2 preprotoxin gene, whose expression is under the control of inducible hybrid promoter GAL-CYC. Markers were used for selection of transformants in appropriate strains. Killer preprotoxin gene confers K2-specific killing and immunity functions.

We determined that the stability of the formed *in vivo* recombinant plasmid pYEXBK (monitored by maintaining the $Ura^+Leu^+K2^+$ phenotype) under both non-selective and leucine-selecting conditions in strains H13 and 21PMR marked by *ura3-52 leu2* mutations reached 94–98% (Table 1, rows 1–5). Quite possibly this high stability level is determined by the presence of a defective *leu2-d* gene, which acts by increasing the copy number of plasmid, while in strains marked only by *ura3-52* mutations (3PMR-1, H14) the stability of plasmid (by $K2^+Ura^+$ phenotype) is lower, ranging from 77 to 83% (Table 1, rows 8, 9) – *leu2-d* in this case is obviously not functioning.

In order to clarify the role of *leu2-d* in the properties of recombinant plasmid, deletion in 3' end of *leu2-d* gene (486bp *EcoR*I-*Bsp*1407I fragment) has been made. Resulted construct pYEXBK-Δleu was propogated in *E. coli* and its integrity confirmed by restriction analysis. *S. cerevisiae* strains, marked by: a) *ura3-52*; b) *leu2*; c) both mutations, were transformed by this plasmid. In b) and c) cases transfor-

Table. **Characteristic of transformants**

No.	Transformants	Phenotype	Number of clones	Stability, %
1	H13-pYEXBK	$K2^+R2^+Ura^+$ Leu^+	1416	97.11 ± 0.45
2	H13-pYEXBK	$K2^+R2^+Ura^+$ Leu^+	978	95.19 ± 0.68
3	21PMR-1-pYEXBK	$K2^+R2^+Ura^+$ Leu^+	2114	97.73 ± 0.32
4	21PMR-1-pYEXBK	$K2^+R2^+Ura^+$ Leu^+	948	95.46 ± 0.67
5	21PMR-1-pYEXBK	$K2^+R1^+R2^+R28^{\pm}$ Ura^+ Leu^+	292	93.84 ± 1.41
6	α'1-pYEXBK	$K2^+R2^+Ura^+$ Leu^+	1062	95.01 ± 0.67
7	α'1-pYEXBK	$K2^+R1^+R2^+R28^{\pm}$ Ura^+ Leu^+	1018	94.99 ± 0.68
8	H14-pYEXBK	$K2^+R2^+Ura^+$	2918	76.97 ± 0.78
9	3PMR-1-pYEXBK	$K2^+R2^+Ura^+$	572	83.22 ± 1.56
10	H13-pYEXBK-Δleu	$K2^+R2^+Ura^+leu^-$	306	58.5 ± 2.82
11	21PMR-1-pYEXBK-Δleu	$K2^+R2^+Ura^+leu^-$	1400	50.71 ± 1.79
12	H14-pYEXBK-Δleu	$K2^+R2^+Ura^+$	1099	88.08 ± 0.98
13	3PMR-1-pYEXBK-Δleu	$K2^+R2^+Ura^+$	727	85.56 ± 1.30

$K2^+R2^+$ – K2 killer phenotype and resistance; leu^-, Leu^+ – auxotrophy and prototrophy by leucine; Ura^+ - prototrophy by uracil.

mants were unable to grow on the minimal media without supplement leucine, what proves the inactivation of *leu2-d* in pYEXBK-Δleu. It was shown that stability of pYEXBK-Δleu plasmid (by means of $K2^+Ura^+$ phenotype) in strains marked by *leu2 ura3-52* mutations (21PMR, H13) has dropped significantly, comparing to original plasmid pYEXBK and reached only 50–58% (table 1, rows 10, 11). It is tempting to conclude that inactivation of *leu2-d* gene was the cause of decreased stability of this plasmid in *ura3-52 leu2* strains. It should be mentioned that in strains marked only by *ura3-52* mutation (3PMR-1, H14) stability of *in vitro* constructed plasmid pYEXBK-Δleu ranges from 85 to 88% (Table 1, rows 12, 13). These results indicate that presence (pYEXBK) or lack of functional *leu2-d* gene (pYEXBK-Δleu) had no impact on the stability of investigated plasmids in *ura3-52* strains.

Expression of killer and immunity phenotypes coded by plasmids pYEXBK and pYEXBK-Δleu has been evaluated in all beforementioned yeast strains – it was find that transformants retains K2 killer and immunity functions. Comparing the properties of plasmids formed *in vivo* (pYEXBK) and *in vitro* (pYEXBK-Δleu) it was mentioned that presence of active *leu2-d* gene (which acts by increasing copy number of plasmids bearing it in the strain) increases K2 type killer toxin expression 1.5–2 times (as observed by evaluating lysis zone on the α'1 strain layer), while the immunity for the toxin does not changes.

Introduction of the pYEXBK plasmid into α'1 *leu2* and 21PMR *ura3-52 leu2* yeast strains and selection by leucine marker leads to a division of the transformants into several phenotypic groups: the first – expressing K2 killer and able to confer immunity to toxins of this type ($K2^+R2^+$) (Table, rows 4, 6); the second – capable to form killing zones on the sensitive strain α'1 and resistant to K1, K2 (and partially to K28) killer toxins ($K2^+R1^+R2^+R28^\pm$) (Table, rows 5, 7) as well as others, different (intermediate) variants of phenotype possessing transformants ($K2^-R2^-Leu^+$; $K2^+R2^+Leu^-$). As is iliustrated by Figure, these groups of transformants differ in colony size. On the other hand, under non-selective conditions (selection by uracil marker) only one type of transformants ($K2^+R2^+$) is obtained (Figure 1). Eliminants formed from the second group of transformants retain the former type of immunity ($R1^+R2^+R28^\pm$), thus proving the role of mutational processes in a strain instead of an impact of genes encoded by plasmid. This is thought to depend on the additive action of killer preprotoxin gene and defective leucine gene, as the transformation of the same strains (α'1 and 21PMR) by the control plasmid pYEX12 (bearing normal *LEU2* marker and K2 preprotoxin gene) and by pYEXBK-Δleu (lacking functional *leu2-d*) leads to the formation of $K2^+R2^+$ transformants alone. In addition, introduction of plasmid pYEXBK-K^- (containing only *leu2-d* as well as *URA3* markers and bearing removed killer preprotoxin gene (deletion of 1.2 kb *Sal*I-*Sal*I fragment)) into these strains determine their ability to grow on the minimal media possessing no killer or immunity phenotype.

In summary, recombination *in vivo* resulted in the formation of pYEXBK, in which the defective *leu2-d* gene determines a high plasmid stability and elevated killer toxin production in *leu2* yeast strains. Combination of both effects (K2 killer and *leu2-d*

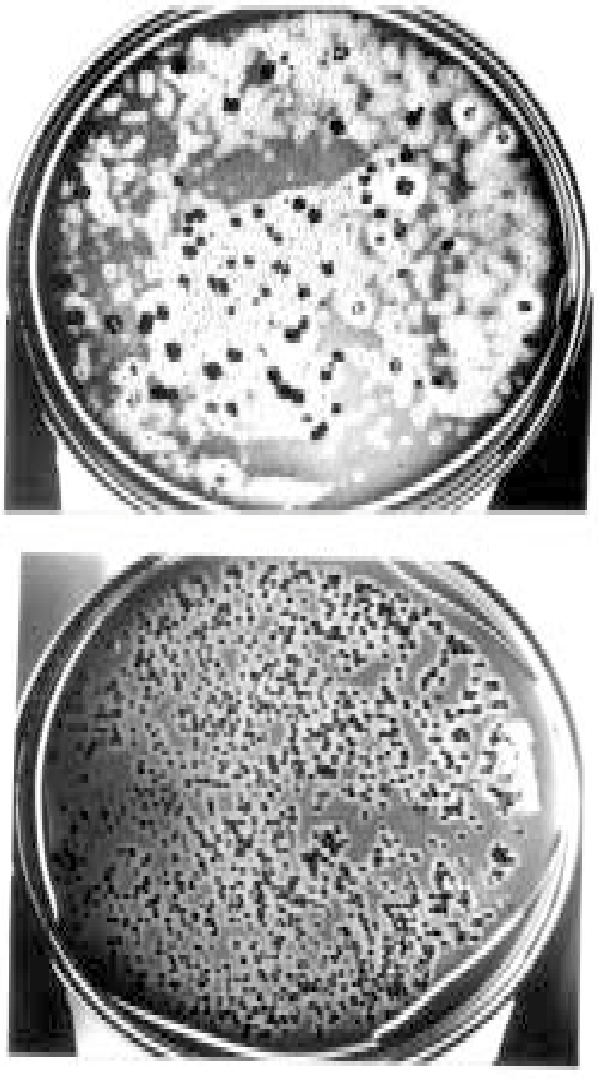

Figure. Transformants of plasmid pYEXBK in 21PMR yeast strain.
1. Selection of transformants by leucine marker: small colonies retain K2 killer and immunity phenotype, big colonies possess $K2^+R1^+R2^+R28^\pm$ phenotype and different variants of intermediate phenotype.
2. Selection of transformants by uracil marker: uniform colonies retain $K2^+R2^+$ phenotype

action) leads to an increased outcome of mutational processes in cells.

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DEFEKTYVAUS LEUCINO GENO *LEU2-D* POVEIKIS REKOMBINANTINĖS PLAZMIDĖS SAVYBĖMS MIELĖSE *S. CEREVISIAE*

S a n t r a u k a

Defektyvus leucino genas *leu2-d*, esantis *in vivo* susiformavusioje plazmidėje pYEXBK, užtikrina aukštą minėtos plazmidės stabilumą (95–98%) tiek selektyviomis, tiek neselektyviomis sąlygomis mielių kamienuose, pažymėtuose *ura3-52 leu2* mutacijomis. Tuo tarpu tik *ura3-52* kamienuose *leu2-d* geno funkcionavimas (pYEXBK) ar inaktyvavimas (pYEXBK-Δleu) nedaro įtakos plazmidžių stabilumui. K2 kilerinio preprotoksino ir defektyvaus leucino genų bendra veikla daro įtaką papildomos transformantų grupės su pakitusiu imuniškumu susidarymą ($K2^{+}R1^{+}R2^{+}R28^{\pm}$). Nustatyta, kad, veikiant *leu2-d* markeriniam genui (lemiančiam padidintą DNR kopijų skaičių), K2 tipo kilerinio toksino ekspresijos lygis pakyla 1.5–2 kartus.