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Expression of *Saccharomyces cerevisiae* K2 preprotoxin gene in *Kluyveromyces lactis* yeast

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Recombinant plasmids pKDSU-K2 and pKDSU-K2-15S2, designed for *S. cerevisiae* K2 preprotoxin gene expression in *Kluyveromyces* and *Saccharomyces* yeast, were constructed. Expression of the K2 killer gene is not regulated by a specific promoter and confers *S. cerevisiae* and *K. lactis* only a weak K2 killer phenotype. The K2 toxin expression regulated by a constitutive phosphoglycerate kinase (PGK1) promoter was found to be efficient and comparable in both yeast species.

Key words: *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, killer toxin, immunity

INTRODUCTION

Besides the value as a model organism in eucaryotic cell biology, yeast species have been developed into an attractive host for the expression, processing and secretion of many recombinant proteins [1]. In a number of studies when identical proteins were expressed in *S. cerevisiae* and other non-conventional yeast, the latter have proven superior both in product yield and in secretion efficiency [2]. Specifically, *K. lactis* has received increasing attention from bioindustries because of GRAS (generally regarded as safe) status [3], molecular genetic accessibility using both integrative and highly stabilized episomal vector systems [1, 2, 4], and excellent fermentative characteristics allowing a large-scale industrial application [2]. Moreover, its capability to export the zymocin (toxin) complex suggests that *K. lactis* is suitable for secretion of large heterologous proteins [2].

S. cerevisiae killer strains secrete a protein toxin derived from the double-stranded RNA (dsRNA) virus [5]. Analysis of corresponding cDNA shows that each virus encodes a single open reading frame for both properties: the precursor of the secreted killer toxin and specific immunity to that toxin [5]. The type of killer depends on the molecular characteristics of the secreted toxin, killing profiles, lack of cross-immunity and the encoded genetic determinants [6]. Among the killer types identified in *S. cerevisiae*, only three (K1, K2 and K28) have been studied in more detail. Physiological studies of the K1 and K2 killer systems suggest that the toxin, an α/β dimmer, binds to β-1,6 D-glucans in the cell wall and perturbs an energized plasma membrane state inducing potassium leakage and cell death [7]. The K28 killer toxin binds to α-1,3 linked mannoproteins and causes early inhibition of DNA synthesis [8]. Immunity is determined by a preprotoxin [9] or internal toxin [10] acting as competitive inhibitors saturating cell membrane receptors that normally mediate the mature toxin action.

Previously we have synthesized, cloned and expressed in yeast the cDNA copy of the *S. cerevisiae* M2 dsRNA virus encoding the K2 killer preprotoxin [11]. In this study, the K2 preprotoxin gene was cloned into the *K. lactis*/*S. cerevisiae* shuttle expression vector, and peculiarities of *S. cerevisiae* K2 killer gene expression in the *K. lactis* yeasts were examined.

MATERIALS AND METHODS

For *S. cerevisiae* K2 preprotoxin gene expression studies, *K. lactis* strains MD2/1(MATα *argA lysA ura3* K^- $pKD1^+$) and MW270-7B (MATa *metA leu2 ura3* K^+ $pKD1^-$), kindly provided by Dr. D. Bartkevičiūtė, as well as the following *S. cerevisiae* strains: 3PMR-1 (*MAT*α *ura3-52 [KIL-0]*); K7 (*MATa arg9 [KIL-K1]*); Rom K-100 (*wt*, *HM/HM [KIL-K2]*); MS300 (*MATa leu2 ura3-52 SKI2-2 [KIL-K28]*) [12] were used. The *E. coli* strain DH5α (*F^- (ϕ80dΔ(lacZ)M15) recA1 endA1 gyrA96 thi1 hsdR17 ($r_k^-m_k^+$) supE44 relA1 deoR* Δ *(lacZYA-argF) U169)* was used for plasmid propagation [13]. Media for the propagation of *S. cerevisiae* and *K. lactis* yeasts as well as standard genetic techniques have been described in Ausubel et al. [14].

The expression plasmids pYEX12 (containing *S. cerevisiae* K2 preprotoxin gene under control of alcoholdehydrogenase ADH1 promoter) [11], pKDSU (multicopy plasmid replicating in bacteria *E. coli* as well as in *S. cerevisiae* and *K. lactis* yeasts) and 15S2 (containing the human insulin gene under control of the *S. cerevisiae*

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phosphoglycerate kinase gene PGK1 promoter) (a generous gift from Dr. D. Bartkevičiūtė) were used for constructing the recombinant plasmids pKDSU-K2 and pKDSU-K2-15S2. General procedures for the construction and analysis of recombinant DNAs were performed as described by Sambrook et al. [15]. All restriction enzymes (*Sal*I, *Eco*RV, *Bsp*68I, *Xho*I, *Hind*III, *Eco*RI), T4 DNA ligase, bacterial alkaline phosphatase, Klenow fragment and DNA size marker (GeneRuler™ DNA Ladder mix) were obtained from UAB Fermentas (Vilnius) and used according to the manufacturer's recommendations.

Transformation of *S. cerevisiae* and *K. lactis* yeast strains was performed using the LiAc/PEG method [16], and the transformants were selected by complementation of *URA3* auxotrophy. The transformants were analyzed for toxin production in a killing zone plate assay following the replica-plating of transformants onto a lawn of the sensitive strain α'1. Immunity was tested by streaking the standard K1, K2 and K28 killer strains on the lawn of transformed cells.

RESULTS AND DISCUSSION

An attractive feature of *K. lactis* yeast to express and efficiently secrete recombinant proteins was used to study *S. cerevisiae* K2 preprotoxin gene expression opportunities and peculiarities in *K. lactis* yeast.

On the basis of the *S. cerevisiae* / *K. lactis* vector system pKDSU, the recombinant plasmid pKDSU-K2 was constructed by inserting the 1200 bp *Sal*I-*Sal*I fragment (bearing *S. cerevisiae* K2 preprotoxin gene) from pYEX12 [11] (Fig. A). It is important to point out that in this construct the preprotoxin gene expression is not regulated by any particular promoter but depends on 5' flanking context. Plasmid pKDSU-K2 was transformed into *S. cerevisiae* 3PMR-1 *ura*3-52 strain and the transformants were selected for uracil auxotrophy. In order to detect the K2 killer phenotype in cells we used indicative MB media (pH 4.0) possessing optimal activity of K2 toxin. Analysis of *S. cerevisiae* strain bearing pKDSU-K2 showed barely detectable killing activity of secreted toxin, and the transformants failed to display the immunity function (Table). They were sensitive to K2 toxins produced by wt K2 killer strains and their own toxin, thus exhibiting a suicide phenotype. These data are in line with previous observations of K2 killer gene features described in [17]. In parallel, *K. lactis* MD2/1 [Kil$^-$] strain was transformed by pKDSU-K2 plasmid, and then a killing zone plate assay was performed. The obtained K2 preprotoxin gene expression level was low, and the observed phenotype was similar in both *K. lactis* and *S. cerevisiae* cells (Table). Unfortunately, we could not test the plasmid-driven K2 type immunity of the *K. lactis* MD2/1 [pKDSU-K2] transformants because of the parental strain's MD2/1 own resistance to all *S. cerevisiae* killers (Table). Thus, there is no possibility to address differences of immunity phenotype in the parental *K. lactis* strain and its transformants. Still, analysis of *S. cerevisiae* 3PMR-1

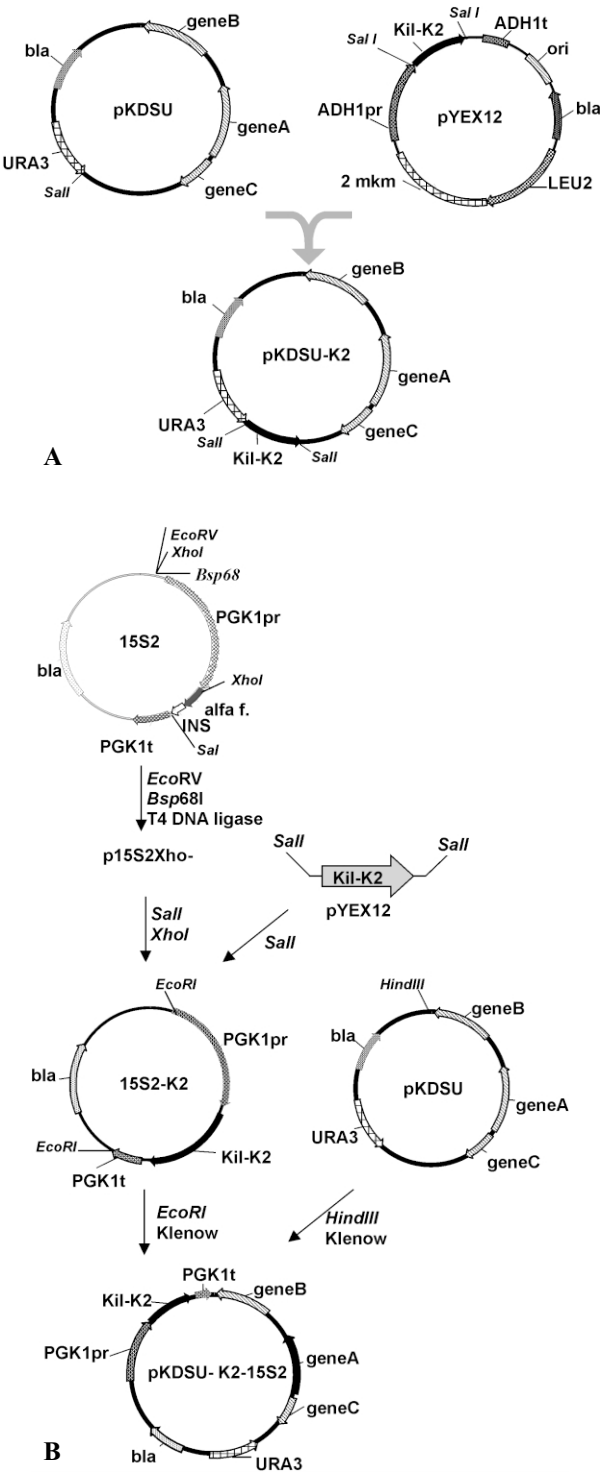

Figure. Construction scheme of pKDSU-K2 and pKDSU-K2-15S2 plasmids

Kil-K2 – *S. cerevisiae* K2 preprotoxin gene; *URA*3, *LEU*2 – genetic markers; 2mkm – sequence originated from 2 μ plasmid of *S. cerevisiae*; bla – gene for β-lactamase; ori – pMB1 replication origin; ADH1pr – alcoholdehydrogenase promoter; ADH1t – alcoholdehydrogenase terminator; PGK1pr – phosphoglycerate kinase promoter; PGK1t – phosphoglycerate kinase terminator; alfa f. – *S. cerevisiae* α-factor signal sequence; INS–insulin gene; geneA, geneB, geneC–*K. drosophilarum* pKD1 plasmid sequence; *Sal*I, *Eco*RI, *Eco*RV, *Bsp*68I, *Xho*I, *Hind*III – restriction endonuclease sites.

Table. **Characteristic of transformants and standard yeast strain**

Yeast strains	K2 killer phenotype	Immunity
S. cerevisiae wt Rom K-100	++++	I2
S. cerevisiae 3PMR-1	–	–
S. cerevisiae 3PMR-1 [pYEX12]	++	I2
S. cerevisiae 3PMR-1 [pKDSU-K2]	±	–
S. cerevisiae 3PMR-1 [pKDSU-K2-15S2]	++	I2
K. lactis MD2/1	–	[I1 I2 I28]*
K. lactis MD2/1 [pKDSU-K2]	+	[I1 I2 I28]*
K. lactis MD2/1 [pKDSU-K2-15S2]	++	[I1 I2 I28]*
K. lactis MW270-7B	–	[I1 I2 I28]*
K. lactis MW270-7B [pKDSU-K2]	+	[I1 I2 I28]*
K. lactis MW270-7B [pKDSU-K2-15S2]	++	[I1 I2 I28]*

The size of clear zones (mm) formed on lawn of sensitive α'1 strain: ++++ (10-9); ++ (5-4); + (2.5-2); ± (1.5-1). I2 – plasmid-driven immunity to K2 type toxin; [I1 I2 I28]* – strain-driven resistance to *S. cerevisiae* K1, K2 and K28 killer toxins.

[pKDSU-K2] agar diffusion assay results, especially the deficiency in K2 type immunity, points to a similar expression of the defective killer preprotoxin unable to confer full immunity in *K. lactis* MD2/1 [pKDSU-K2] cells, either.

In order to achieve a higher level of K2 toxin production in *K. lactis* yeast we created a new dual expression plasmid, pKDSU-K2-15S2, where killer gene expression is controlled by the of constitutive *S. cerevisiae* phosphoglycerate kinase gene (PGK1) promoter. First, one of two *Xho*I sites was eliminated from 15S2 plasmid by deleting the small 33 bp *Eco*RV–*Bsp*68I fragment (resulting in plasmid p15S2 Xho-). The K2 preprotoxin gene was prepared in the same way as described before and ligated into the p15S2 Xho- vector linearized by *Sal*I and *Xho*I restriction enzymes, resulting in 15S2-K2. The *S. cerevisiae*/*K.lactis* shuttle vector pKDSU was prepared by using *Hind*III restriction and Klenow enzymes, followed by ligation with the 3000 bp insert obtained from 15S2-K2 plasmid (treated by *Eco*RI restriction and Klenow enzymes) (Fig.1B). Extensive restriction analysis of the resulting plasmid pKDSU-K2-15S2 confirms the presence of essential elements for replication in *S. cerevisiae* and *K. lactis* yeasts as well as the K2 preprotoxin gene inserted between the PGK1 promoter and terminator. The new construct was introduced into *S. cerevisiae* 3PMR-1 and *K. lactis* MD2/1 strains and their killing capabilities were tested. In case of *S. cerevisiae* 3PMR-1 [pKDSU-K2-15S2] transformants, we observed a ~4 times higher K2 toxin expression level as compared with the construct-lacking promoter sequence and similar to expression of the K2 preprotoxin gene subjected to the control of the constitutive ADH1 promoter (Table). In case of *K. lactis* MD2/1 [pKDSU-K2-15S2] transformants, it was expected to reach the highest K2 toxin production. The reason for this was the ability of *K. lactis* to produce functional foreign proteins in big amounts [2]. However, the observed killing ability of *K. lactis* strains was similar to an analogous plasmid-driven killer phenotype in *S. cerevisiae* and half as high in comparison with the *S. cerevisiae* K2 toxin producing the wild type strain Rom K-100 (Table). In parallel, we transformed the pKDSU-K2-15S2 plasmid into another *K. lactis* strain, MW270-7B [Kil^+] possessing the *K. lactis* killer phenotype, and essentially the same *S. cerevisiae* toxin expression level was observed which was independent of the presence of *K. lactis* own toxin (Table).

In summary, it is concluded the that plasmid-coded *S. cerevisiae* K2 preprotoxin gene controlled by constitutive ADH1 or PGK1 promoters ensures a comparable level of toxin in both *S. cerevisiae* and *K. lactis* cells.

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O. Černyšova, E. Servienė, V. Melvydas

SACCHAROMYCES CEREVISIAE K2 PREPROTOKSINO GENO EKSPRESIJOS *KLUYVEROMYCES LACTIS* MIELĖSE TYRIMAS

Santrauka

Sukonstruotos pKDSU-K2 ir pKDSU-K2-15S2 plazmidės, tinkamos *S. cerevisiae* K2 kilerinio geno funkcionavimo tyrimams *Saccharomyces* ir *Kluyveromyces* mielėse. Nustatyta, kad specifiniu promotoriumi nereguliuojama K2 preprotoksino geno raiška tiek *S. cerevisiae*, tiek *K. lactis* kamienuose užtikrina silpną kilerinį fenotipą. Tuo tarpu konstitutyvus fosfogliceratkinazės promotorius palaiko aukštą K2 preprotoksino ekspresijos lygį abiejų rūšių mielėse.