Influence of carbon source on the expression of the *S. cerevisiae* K2 killer preprotoxin gene

E. Servienė,
A. Lebionka,
V. Melvydas

Institute of Botany,
Žaliųjų ežerų 49,
LT-2021 Vilnius, Lithuania

Analysis of the *Saccharomyces cerevisiae* K2 killer gene expression profile revealed a novel feature for a strain to express different phenotypes, depending on the origin of carbon source in the growth media. The promoter-specific sugar, galactose, confers both killer and immunity properties to a strain possessing plasmid pBK, where preprotoxin gene expression is controlled by the of GAL-CYC1 promoter; while non-specific glucose is able to confer a killer and partially a suicide phenotype. It was determined that under promoter-inducing conditions the strains tested produced a twofold higher level of secreted killer toxin as compared to non-inducing conditions. When the same killer preprotoxin gene is controlled by the of ADH1 promoter (in the case of plasmid pX12), change of growth conditions from inducing into non-inducing leads to a decrease of toxin activity (2.5-fold) retaining the full immunity to K2-type killers.

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

INTRODUCTION

The widespread killer phenomenon in yeast is based on the secretion of protein or glycoprotein toxins (killer toxins) lethal to sensitive strains of the same species and a wide spectrum of other yeast genera [1]. In *Saccharomyces cerevisiae*, three different killer toxins (K1, K2 and K28) have been clearly identified, all genetically encoded by double-stranded M dsRNA "killer" viruses stably persisting within the cytoplasm of the infected host cell [2]. Analysis of the corresponding cDNAs show that each virus encodes a single open reading frame for both the precursor of the secreted killer toxin and specific immunity to that toxin properties [2–4].

Physiological studies of the best-characterized K1-type killer system suggest that the toxin perturbs an energized plasma membrane state inducing potassium leakage and subsequent cell death [5]. Following one of the hypotheses, immunity is determined by a precursor that can act as a competitive inhibitor saturating cell membrane receptors that normally mediate the mature toxin action [6].

The structure and action of protein toxin produced by the K2 killer system has not yet been examined in detail. In this work, the gene for K2 killer toxin was subjected to expression studies in *S. cerevisiae* where strains featuring constructs containing an appropriate promoter display different phenotypes depending on the carbon source in growth media.

MATERIALS AND METHODS

The following *S. cerevisiae* strains were used in this work: α'1 (*MATαleu2-2 [KIL-0]*) [7]; Rom K-100 (*wt*, *HM/HM [KIL-K2]*) [8]; M437 (*HM/HM [KIL-K2]*) [9]; 3PMR-1 (*MATα ura3-52 [KIL-0]),* 21PMR (*MATα ura3-52 leu2 [KIL-0]),* H13 (*MATa ura3-52 leu2 [KIL-0]*) [10]. 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 [11].

Plasmid pX12 code for a *LEU2* marker gene as well as K2 killer preprotoxin gene (under the control of ADH1 promoter) [12]; plasmid pBK contains *URA3*, *leu2-d* and K2 cDNA (under the control of GAL-CYC1 promoter) [13].

Media for the growth of *S. cerevisiae* as well as standard genetic techniques were as described in Ausubel et al. [14]. Transformation of *S. cerevisiae* was performed by non-spheroplasting lithium chloride procedure of Ito et al. [15]. Transformants were selected by complementation of auxotrophic markers; clones were verified for toxin production by replica plating to a lawn of *S. cerevisiae* α'1 strain. Transformation of *E. coli* was performed following a standard $CaCl_2$ procedure [14].

K2-specific killing and immunity phenotypes were determined in an agar diffusion assay on the methylene blue (MB) agar plates [16]. Toxin activity in culture supernatant (obtained by filtration

through PVDF membrane) was quantified by pipetting 100 µl samples into wells (10 mm in diameter) cut in an MB plate seeded with the sensitive α'1 yeast strain (~10^6 cells per plate) and incubated at 18 °C for 72 h. The diameter of the growth-free zone around the wells was proportional to the logarithm of the killer toxin activity expressed in arbitrary units (U/ml) [17].

RESULTS AND DISCUSSION

Plasmid pBK contains a functional gene for K2 killer preprotoxin, controlled by GAL-CYC1 (galactose-specific) promoter [12]. This construct was introduced into the sensitive strains 3PMR-1 *ura3-52*, 21PMR *ura3-52 leu2* and H13 *ura3-52 leu2*. Transformants selected by the complementation of *URA3* auxotrophy were tested for the immunity and killing capabilities using the indicative media (MB) with galactose or glucose as a carbon source (Fig. 1A). In the case with galactose, strains bearing a recombinant plasmid showed a K2 killer phenotype and resistance to killer toxins of the same type. Alternatively, when glucose was used as a carbon source, transformants were able to kill a sensitive α'1 strain (Fig. 1A) being sensitive to K2 wild type killer and only partially resistant to its own toxin.

To estimate the level of produced toxin, transformants were grown for 96 h in liquid rich media with 1) galactose or 2) glucose as a carbon source. It was determined that under GAL-CYC1 promoter-inducing conditions (1) the strains produced high levels of secreted killer toxin (21PMR[pBK] – 69.3 ± 5.4 U/ml; 3PMR-1[pBK] – 66.2 ± 5.4 U/ml; H13[pBK] – 61.2 ± 3.2 U/ml), while under non-inducing conditions (2) a moderate level of toxin (21PMR[pBK] – 33.2 ± 2.7 U/ml; 3PMR-1[pBK] – 34.7 ± 2.7 U/ml; H13[pBK] – 30.7 ± 1.6 U/ml) was produced (Fig. 1B). The wild type K2 killer expressing the strain Rom K-100 produces a somewhat higher level of killer toxin growing in media with either glucose or galactose (122.3 ± 6.4 U/ml) (Fig. 1B). So, the transformants encompassing plasmid pBK are able to produce killer toxin in amounts of ~50% under inducing or ~25% under non-inducing conditions compared to the wild type strain Rom K-100 production level.

Parallelly, the immunity of pBK transformants was investigated by evaluation of the amount of survived cells subjected to the externally applied K2 toxin. 3PMR-1 *ura3-52*, 21PMR *ura3-52 leu2* and α'1*leu2*

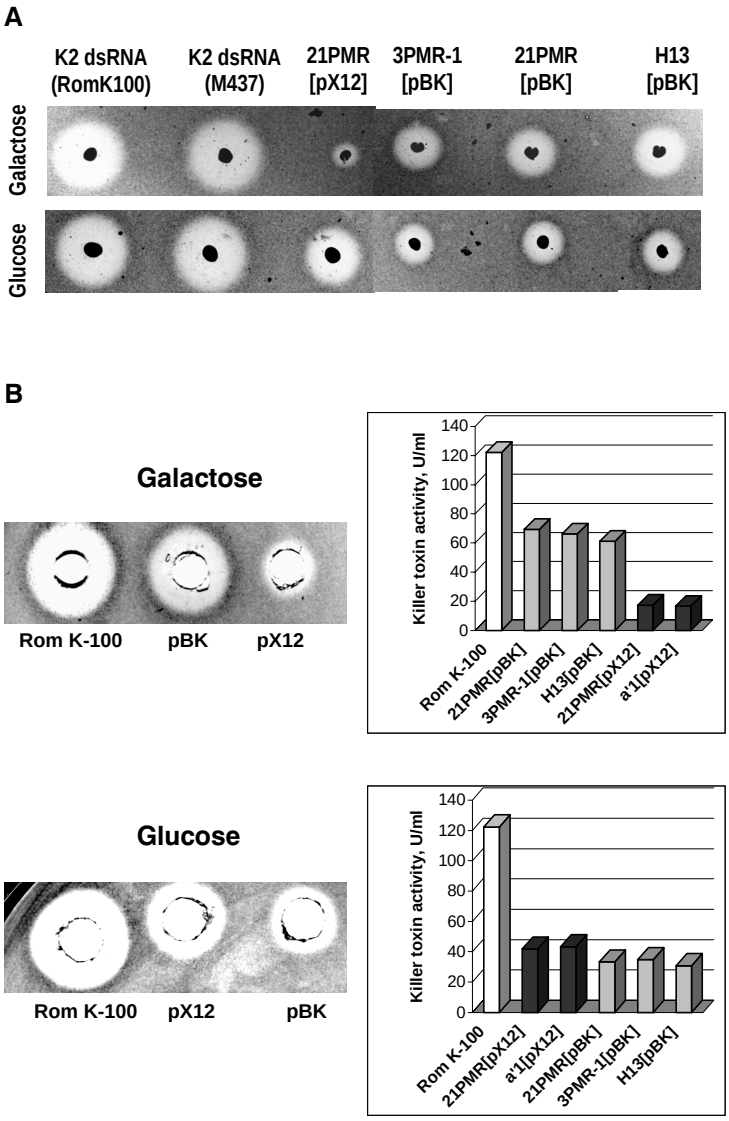

Fig. 1. Activity of K2 killer toxin in strains encompassing pBK and pX12 plasmids along with wild type strains Rom K-100 and M437. A. Killer property of M2 dsRNA *wt* killer strains (Rom K100 and M437) and K2 preprotoxin gene-bearing (pBK and pX12) recombinants. The K2 killer phenotype was determined by patching of control M2 dsRNA-containing killer strains and yeast strains bearing pBK and pX12 onto MB agar plates (pH 4.0), using galactose or glucose (as indicated on the left side) as a carbon source seeded with sensitive *S. cerevisiae* strain α'1(~10^6 cells per plate).
B. Left: K2 toxin activities determined in supernatants of cultures by pipeting 100 µl samples into wells (10 mm in diameter) cut into MB agar plates seeded with a sensitive α'1 yeast strain (~10^6 cells per plate) and incubating the plates at 18 °C for 72 h. Right: diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity, which is expressed in arbitrary units (U/ml)

strains bearing pBK were incubated for 18 h at 18 °C in liquid minimal media (with either glucose or galactose as a carbon source) containing 42 U/ml of K2 toxin. It was determined that under inducing conditions (in the presence of galactose) 88÷93% of cells survived (Fig. 2, left). This observation proves an expression of a sufficient amount of preprotoxin (or mature toxin) to immunize these cells. When the carbon source is changed to glucose, the number of survived cells drops to 11÷15% (Fig. 2, right) (compare to 4÷8% of survived cells of K2-null strains α'1, 3PMR-1 and 21PMR). However, as is mentioned before, killer toxin activity in pBK-bearing strains under the non-inducing conditions decreases only ~2 times. Thus, in case of non-inducing conditions the resulting protein was unable to confer even a moderate level of immunity to the external K2 toxin. The latter result gives another proof of compromised immunity of the strain under described conditions made from agar diffusion assay (see above).

Analysis of the expression of the K2 killer preprotoxin gene subjected to the control of ADH1 promoter (plasmid pX12) displays different results (Fig. 1). 21PMR[pX12] and α'1[pX12] transformants tested under either inducing (glucose) of non-inducing (galactose) conditions demonstrated both K2 killer and immunity phenotypes. Supernatant fractions showed the same biological specificity as the parental strains (Fig. 1B). Secreted K2 killer toxin was barely detectable in the supernatant from cultures of the strains containing pX12 plasmid grown in media with galactose (21PMR[pX12] – 17.2 ± 2.4 and α'1[pX12] – 16.6 ± 1.3 U/ml), whereas, supernatants from the same transformants (grown in the liquid media supplied with glucose) produced considerably stronger signal (toxin activity 21PMR[pX12] – 41.8 ± 3.4 and α'1[pX12] – 43.2 ± 5.9 U/ml). Comparison of the amounts of the produced toxin in Rom K-100 and the mentioned strains indicates that even under inducing conditions recombinant strains are able to produce only 33% of a wild-type strain toxin (Fig. 1B). At the same time, it was demonstrated that survival of a recombinant strain bearing pX12 apparently did not depend on the carbon source in growth media – 85÷87% of cells survived under the promoter-inducing conditions *versus* 80÷82% under non-inducing conditions. As is shown in Fig. 1, change of carbon source to non-specific led to a ~2.5 fold decrease of toxin activity in pX12 transformants. Despite this decrease in toxin activity, the immunity component is (still) able to mask efficiently receptor sites from the toxin action.

The toxin amounts produced under non-inducing conditions by strains bearing pBK (30.7÷34.7 U/ml) were twofold higher as compared to pX12-containing strains (16.6÷17.2 U/ml). Following one of the hypotheses, an effective masking of receptor sites may require a minimum number of immunity-conferring molecules [12]. Interestingly, the immunity function fully protects pX12 transformants with a lower production of killer. This apparent disagreement may result from the properties of GAL-CYC1 functioning under non-inducing conditions, namely, alternative transcription of the killer gene. The reason for the inability to immunize cells by the toxin produced by a pBK-bearing strain is currently under further investigation.

In summary, it could be concluded that the plasmid-containing K2 preprotoxin gene under the control of ADH1 promoter produces a toxin able to immunize cells under both inducing and non-inducing conditions. In clear contrast, subjecting of K2 preprotoxin gene to the control of GAL-CYC1 promoter ensures a considerably higher toxin production in the presence of either of carbon sources, although this time non-inducing sugar leads to a compromised immunity of cells to the action of K2 killer toxin.

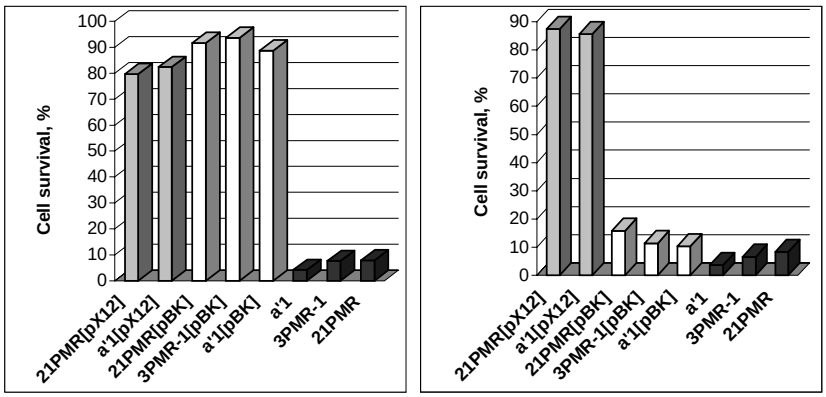

Fig. 2. Survival of pBK and pX12-bearing transformants in presence of externally applied K2 killer toxin.
Strains are indicated on the abscisse. Cell survival estimated in % as a ratio of cells grown in presence (42 U/ml) and absence of externally applied K2 killer toxin

References

1. Heintel T, Zagorc T, Scmitt MJ Appl Microbiol Biotechnol 2001; 56: 165–72.
2. Wickner R Microbiol Rev 1996; 60 (1): 250–65.

3. Tipper DJ, Schmitt MJ Mol Microbiol 1991; 5: 2331–38.
4. Schmitt MJ, Eisfeld K Recent Res Devel Virol 1999; 1: 525–45.
5. Ahmed A, Sesti F, Ilan N et al. Cell 1999; 99: 283–91.
6. Boone C, Bussey H, Greene D et al. Cell 1986; 46: 105–13.
7. Čitavičius D, Inge-Večtomov S. Genetica (Russian) 1972; 1: 95–102.
8. Jokantaitė T, Laurinavičienė D, Bistrickaitė G Abstracts of 11th International Conference on Yeast Genetics and Molecular Biology, Montpellier, France, 1982: 47.
9. Naumova TI, Naumov GI Genetica (Russian) 1973; 9: 85–90.
10. Servienė E, Melvydas M Biologija 2001; 4: 30–3.
11. Woodcock DM, Crowther PJ, Droherty J et al. Nucleic Acids Ress. 1989; 17 (9): 3469–78.
12. Meškauskas A, Čitavičius D. Gene 1992; 111: 135–9.
13. Servienė E, Melvydas V. Biologija 1999; 2: 14-7.
14. Ausubel FM. Current Protocols in Molecular Biology. John Wiley and Sons, Inc., NY, 1999.
15. Ito H, Murata K, Kimura A. J Bacteriol 1983; 153: 163–8.
16. Leibowitz MJ, Wickner RB. Genetics 1976; 82: 429–42.
17. Schmitt MJ, Tipper DJ. Mol Cel Biol 1990; 10: 4807–15.

E. Servienė, A. Lebionka, V. Melvydas

ANGLIES ŠALTINIO POVEIKIS MIELIŲ *S. cerevisiae* K2 PREPROTOKSINO GENO EKSPRESIJAI

Santrauka

Mielių *S. cerevisiae* K2 preprotoksino geno, kontroliuojamo GAL-CYC1 promotoriumi, ekspresijos tyrimai atskleidžia skirtingas fenotipo išraiškas priklausomai nuo terpėje esančio anglies šaltinio. Specifinis promotoriui substratas – galaktozė sąlygoja tiek kilerinę, tiek rezistentiškumo funkciją, tuo tarpu nespecifinis substratas – gliukozė nulemia kilerinį ir iš daliesi savižudišką fenotipą. Nustatyta, kad indukuojančiomis sąlygomis pBK transformantų sekretuojamas kilerinio toksino lygis (61,2÷69,3 U/ml) dukart viršija toksino sekreciją neindukuojančiomis sąlygomis (30,7÷34,7 U/ml). Tuo tarpu transformantai, turintys pX12 plazmidę (kilerinio preprotoksino genas kontroliuojamas ADH1 promotoriumi), produkuoja 41,8÷43,2 U/ml toksino terpėje su gliukoze ir 16,6÷17,2 U/ml terpėje su galaktoze, tačiau abiem atvejais visiškai išlaikoma imuniniškumo funkcija.