Employment of deleterious genes for enhancing the production of *Saccharomyces cerevisiae* K2 toxin

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Ligation of two deleterious *Saccharomyces cerevisiae* K2 preprotoxin genes resulted in formation of constructs able of elevating protein production to the level comparable to that of the *wt* strain Rom K-100. The lack of the first start codon in the preprotoxin gene detains the immunity function of the resulting protein; this effect is partially reversed by the duplication of the truncated gene.

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

INTRODUCTION

Killer strains of *S. cerevisiae* secrete a protein toxin derived from the double-stranded RNA (dsRNA) virus [1]. These toxins are able to kill non-killer yeasts as well as yeasts belonging to the different killer classes while the toxin-producing cells remain intact [1, 2]. The type of killer depends on the molecular characteristics of the secreted toxins, killing profiles, lack of cross-immunity and the encoded genetic determinants [2]. Among the killer types identified in *S. cerevisiae,* only three (K1, K2 and K28) have been studied in more detail.

The K2-type killer preprotoxin cDNA has been synthesized from the M2-1 dsRNA fragment and its nucleotide sequence determined [3]. The K2 killer preprotoxin is synthesized as a 362-amino acid precursor, which consists of a N-terminal secretion signal followed by the two toxin subunits, α – 172 aa and β – 140 aa long [3, 4]. Expression of the precursor gene cDNA on a multicopy plasmid proved the encoding of both the immunity and the toxin activities by a single precursor, ORF [3]. Site-specific mutagenesis of the K2 toxin gene identified one of the two potential KEX2 cleavage sites to be critical for the toxin action [4]. Changes in both signal and subunit sequences have been demonstrated to compromise or abolish the immunity function of the resulted protein [5].

In this paper, we show that the derivative containing two fused K2 preprotoxin genes without a first start codon elevates the production of killer toxin to the level similar to that of the wild-type killer strain. Investigation of the immunity of such constructs is also presented.

MATERIALS AND METHODS

The Plasmid pBK containing *URA*3, *leu*2-d and K2 cDNA (under the control of GAL-CYC1 promoter) [6] was used for the construction of plasmids bearing two truncated K2 preprotoxin genes. The following *S. cerevisiae* strains were used in this work: α′1 (*MAT*α *leu2-2 [KIL-0]*); Rom K-100 (*wt*, *HM*/*HM [KIL-K2]*); M437 (*HM*/*HM [KIL-K2]*); 3PMR-1 (*MAT*α *ura3-52 [KIL-0]*), 21PMR (*MAT*α *ura3-52 leu2 [KIL-0]*) [7]. The *E. coli* strain DH5α was used for plasmid propagation [8].

The media for growing *S. cerevisiae* as well as standard genetic techniques were as described in Ausubel [9]. K2-specific killing and immunity phenotypes, toxin activity in the supernatant were determined in an agar diffusion assay on the methylene blue (MB) agar plates [10]. 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) [10]. The immunity of a strain was estimated by evaluating the amount of viable cells in media containing 42 U/ml of the killer toxin (treatment at 18 °C for 24 h) *versus* the total number in killer-free media.

RESULTS AND DISCUSSION

Analysis of the killing activity of strains bearing the pBK (K2 preprotoxin gene under the control of GAL-CYC1 promoter) showed that this construct was unable to achieve the K2 dsRNA-containing wild strain level of toxin expression [11]. Plasmids with a duplicated full-length killer preprotoxin gene were designed in order to enhance killer toxin production.

However, all attempts to obtain such a construct failed. Taking advantage of a *Bam*HI site situated downstream from the first initiation codon, deletion of the 27 bp fragment upstream the killer gene and 5 bp of the coding sequence have been introduced (construction pBK4 – a deleterious killer gene in direct orientation to GAL-CYC1 promoter and pBK4r – in reverse orientation). Plate tests indicate that deletion of the first start codon of the preprotoxin gene results in a clear decrease in toxin ($K2^{\pm}$) secretion (Fig. 1, A) and inactivates the immunity (K2 killers form killing zones on the lawn of pBK4 or pBK4r transformants) (Fig. 2, A). It was determined that strains bearing the pBK4 plasmid under the GAL-CYC1 promoter inducing conditions (galactose) produce low levels of a killer toxin (activity 15.5 ± 0.7 U/ml), and under the non-inducing conditions (glucose) the toxin is hardly detectable at all (Fig. 1, B). 3PMR-1[pBK4r] transformants are not influenced by the carbon source in means of toxin activity – it remains low in both cases (12.6 ÷ 13.1U/ml) (Fig. 1, B). Toxin activity tests indicate that pBK4 and pBK4r-bearing strains produce approximately a 10-fold lower amount of toxin as compared to the wild-type killer strain Rom K-100 and 4-fold *versus* strains bearing pBK (native killer gene).

Parallelly, the immunity of strains bearing a truncated preprotoxin gene were investigated by evaluating the amount of alive cells subjected to the externally applied K2 toxin. Under the induced conditions (in the presence of galactose) only 24.7 ± 4.1% of 3PMR-1[pBK4] cells were found to survive. When the carbon source was glucose, the number of viable cells dropped to 9.1 ± 0.7% (Fig. 2, C). At the same time, it was demonstrated that the survival of 3PMR-1[pBK4r] transformants remained low (12.7 ÷ ÷ 13.9%) and did not depend on the carbon source in growth media (Fig. 2, C). It was shown that the toxin and the immunity component in this subtype of killers are encoded by the same ORF [3, 4]. As there are several potential in-frame initiation codons at the beginning of the ORF, the exact start of translation is uncertain. Previously, most 5′ ATG have been predicted as the initiation codon for protein synthesis [4]. The obtained results (on pBK4 and pBK4r) indicate that some level of killer preprotoxin expression from the further but first initiation codon(s) may be enough for a killer but not for immunity function. Therefore the unaltered start of the killer toxin gene for the formation of full-value immunity is indispensable.

Constructs containing fused two such deleterious killer genes (pBK5 and pBK5r) have been obtained. Both pBK5 (two colinear Kil-K2 genes lacking the first start codon in direct orientation to the GAL-CYC1 promoter) and pBK5r (the same killer genes both in

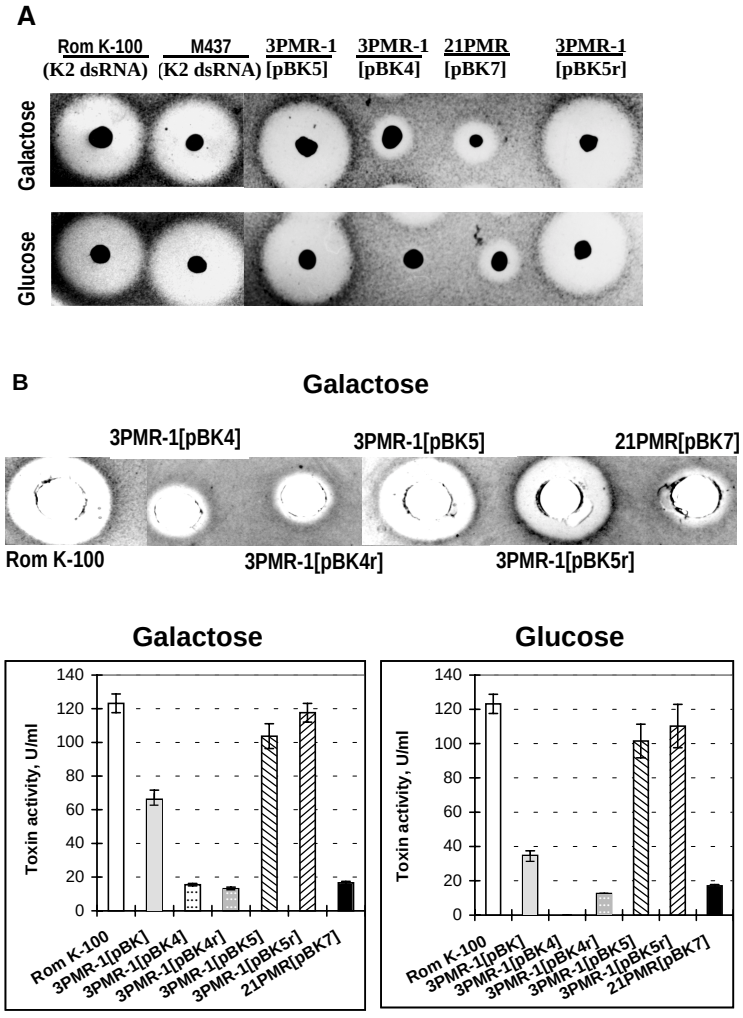

Fig. 1. Killer property of M2 dsRNA wild type killer strains (Rom K-100 and M437) and truncated K2 preprotoxin gene-bearing constructs

A K2 killer phenotype was determined by patching the control M2 dsRNA-containing killer strains and deleterious pBK variant-bearing yeast strains onto MB agar plates (pH 4.0) using galactose or glucose as a carbon source, seeded with a sensitive *S. cerevisiae* strain, α′1 (10^6 cells per plate). pBK4, pBK4r, pBK5 and pBK5r were expressed in strain 3PMR-1; pBK7 was expressed in strain 21PMR.

B K2 toxin activities determined in supernatants of cultures by pipetting 100 µl samples into wells (10 mm in diameter) cut into MB agar plates, seeded with a killer-sensitive α′1 yeast strain (~10^6 cells per plate) and incubating the plates at 18 °C for 72 h. The diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity expressed in arbitrary units (U/ml)

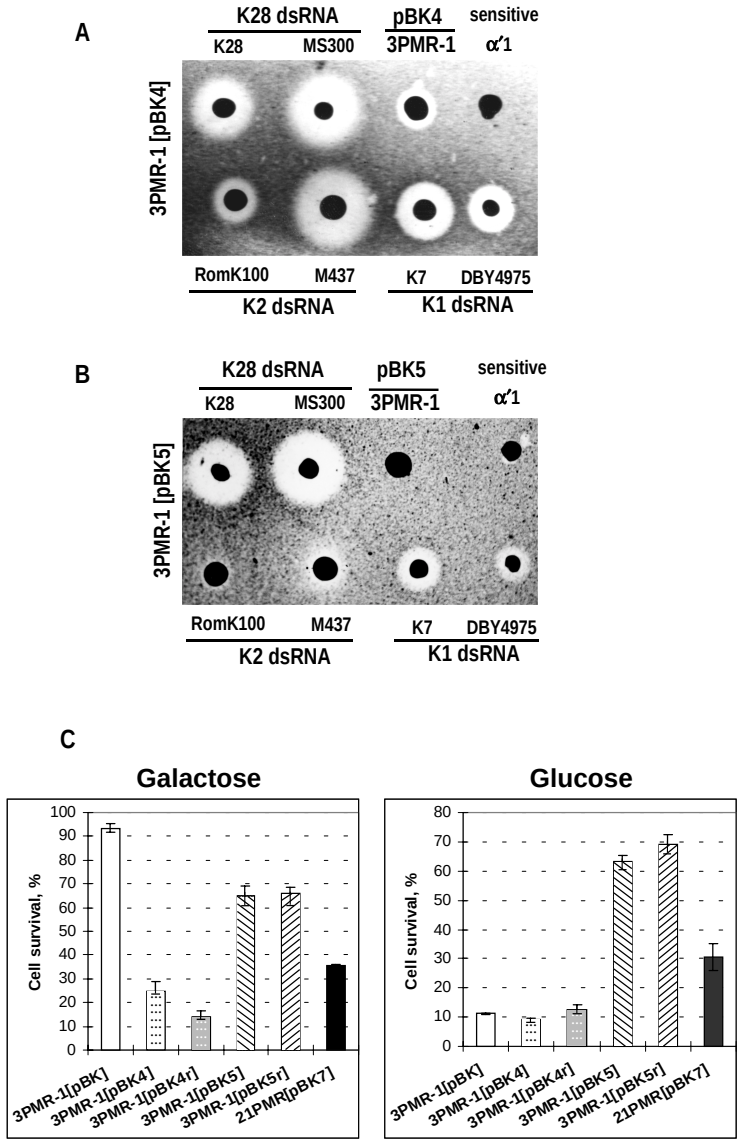

Fig. 2. Immunity function of truncated killer gene-bearing constructs

The transformants were tested for immunity using MB media (pH 4.8) with galactose as a carbon source. Similar amounts of **A.** 3PMR-1[pBK4] and **B.** 3PMR-1[pBK5] strain cells were seeded into MB plates. Test killer strains, transformants of interest and the killer-sensitive strain α′1 were patched onto the plates. Constructs from the corresponding strain used in the seeding of plates are bolded. **C** Cell survival estimated in % as a ratio of cells grown in presence (42 U/ml) and absence of externally applied K2 killer toxin

reverse orientation to the promoter) harbouring strain 3PMR-1 express an increased K2 killer toxin production (Fig. 1, A, B) and now exhibit a partially immune phenotype (Fig. 2, B). The wild K2 killer-expressing strain RomK-100 does not kill these transformants, while the other wild killer strain, M437 forms small killing zones in plate tests. Surprisingly, the tested strains (3PMR-1 featuring pBK5 or pBK5r) formed an inhibition zone on the lawn of the killer-sensitive yeast strain α′1 similar in size to that of the dsRNA-containing wild killer strain RomK100 (the donor of M2 dsRNA, used for cDNA synthesis) and approximately 4 times bigger as compared to those formed by transformants harbouring pBK4 (bearing one truncated preprotoxin gene) (Fig. 1, A). The activity of the secreted K2 killer toxin in the supernatants from 3PMR-1[pBK5] and 3PMR-1[pBK5r] cultures reach 101.5 ÷ 117.6 U/ml, while the *wt* strain Rom K-100 shows a toxin activity of 123.1 ± 5.6 U/ml (Fig. 1, B). It was determined that the survival of strains bearing pBK5 (63.4 ÷ 65.1%) as well as pBK5r (66.0 ÷ 68.9%) was moderate and apparently did not depend on the carbon source in growth media. Plasmids pBK5 and pBK5r were propagated in *E. coli*, the subsequent retransformation into the initial strain 3PMR-1 *ura3-52* confirmed the described phenotype. Transformants bearing any of both plasmids expressed an increased K2 killer production level on galactose as well as glucose-containing media (Fig. 1, B). These results indicate that the high level of killer preprotoxin expression is poorly influenced by the GAL-CYC1 promoter itself, depending on sequences inside the coding part of the killer preprotoxin gene or its close 3′ surrounding. To prove the latter idea, we have obtained a pBK-based construct, pBK7 (containing 3′ termini of the K2 precursor gene with a termination sequence (126 bp in total) fused to killer preprotoxin gene lacking the first start codon), in which the GAL-CYC1 promoter is omitted. However, strain 21PMR bearing this plasmid expressed a low level of toxin activity (16.6 ÷ 16.9 U/ml) (Fig. 1 A, B) and was sensitive to K2 killer toxins (30.5 ÷ 35.5% cells survived in the test of treatment with an external toxin).

It is tempting to conclude that the high level of toxin protein production provided by pBK5 or pBK5r is closely linked with the gene dose effect realized by simultaneous expression of both colinear genes or even by the influence of inner sequence properties of the first killer gene on the expression of the linked second gene. Strains bearing these plasmids not only express increased killer toxin production, but also partially restore the immunity, even when the construct employs a defective precursor gene (the resulting protein was unable to confer full immunity). The partial recovery of immunity function by an increased level of killer toxin points to participation of the killer protein in the immunization mechanism. Investigation of re-

combinant plasmids ensuring the elevated levels of toxin protein will shed further light on the functioning of the K2 killer system – a close connection of killing and immunity profiles.

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PAŽEISTAS GENAS IR PADIDINTA *S. CEREVISIAE* K2 TOKSINO PRODUKCIJA

S a n t r a u k a

Sukonstruotos rekombinantinės plazmidės, kuriose sujungti du deleciniai (be pirmųjų ATG kodonų) *S. cerevisiae* K2 preprotoksino genai, išsidėstę tiesioginėje ir GAL-CYC1 promotoriaus atžvilgiu atvirkštinėje orientacijoje. Nustatyta, kad šių konstrukcijų transformantai išskiria padidintą toksino kiekį, prilygstantį laukiniam mielių kamienui Rom K-100. Pirmojo starto kodono pašalinimas preprotoksino gene sąlygoja savižudišką fenotipą, tuo tarpu padidinus pažeisto geno dozę (suliejant iš eilės du pažeistus genus) iš dalies atstatoma imuninė funkcija.