# Influence of yeast K2 killer preprotoxin gene mutations on the phenotype of transformants

**G. Gulbinienė,**
**T. Jokantaitė,**
**V. Melvydas**

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

The killer preprotoxin gene in yeasts is a code of both killer and immunity phenotypes. The properties of yeast transformants containing mutations in the N-terminal leader of preprotoxin have been investigated. The leader of preprotoxin was involved in forming of immunity – mutations inactivated the immunity without totally eliminating toxin activity. It was demonstrated that secretion of killer toxin may occur in the absence of ADH1 promoter – transformants containing these constructs were able to kill sensitive strains and thus made an active toxin, however, they exhibited a greatly reduced immunity.

**Key words:** *Saccharomyces cerevisiae*, killer toxin, site-directed mutagenesis, immunity

## INTRODUCTION

In the yeast species *Saccharomyces cerevisiae* there are strains which can kill sensitive strains of the same or different yeast species. These killer strains have been classified into three main groups (K1, K2 and K28) on the basis of the molecular characteristics of the secreted toxins, their killing profiles, the lack of cross-immunity, and the encoding genetic determinants [1]. The K1, K2 and K28 toxins are encoded by different cytoplasmically inherited satellite dsRNA (M1, M2 and M28), encapsidated in virus-like particles and dependent on another group of helper yeast viruses (L-A) for their replication and encapsidation [2]. Even though the toxins have different amino acid compositions and modes of molecular action, they show some general characteristics in their mechanisms of synthesis, processing, and secretion. Each toxin is synthesised as a single polypeptide preprotoxin comprising potential KEX2/KEX1 cleavage and N-linked glycosylation sites. The preprotoxins have similar overall structures and, once synthesised, undergo posttranslational modifications resulting in secretion of the mature, active toxin [3].

The K2 killer preprotoxin is a glycosylated protein which consists of an N-terminal leader followed by two toxin subunits, α and β. The extracellular toxin is an α/β dimer with some disulphide bonds. Two subunits of the mature toxin are produced from the preprotoxin by the action of proteases KEX1 and KEX2. The expression of the precursor gene cDNA has shown that both the immunity and toxin activities are encoded on the precursor gene [4]. The K2 killer system is one of the many described among the yeasts, although the structure and action of the protein toxins produced by these killers have not been examined in any detail. The aim of the present study was to find, through site-directed mutagenesis, the role of leader peptide in secretion of killer toxin and formation of immunity. In addition, the dependence of preprotoxin gene expression upon ADH1 promoter has been investigated.

## MATERIALS AND METHODS

The heterotalian *S. cerevisiae* strain α'1 (*MATα leu2* (KIL-0)) [5] was transformed according to [6]. Transformants were selected by complementation of *LEU2* auxotrophy. Clones were checked for toxin production in a killing zone plate assay following replica-plating of transformants onto a lawn of sensitive strain α'1. Immunity was tested by streaking the standard killer strains on the lawn of transformed cells. Killer phenotype-selective indicative media [7] containing 0.2 M Na phosphate-citrate buffer pH 4.8 and methylene blue was used to test killer toxin production and the immunity of transformants. For the stability test, transformants from selective media [8] were recloned on YEPD [8] and grown for 3 days at a temperature of 30 °C with subsequent replication on the indicatory plates. The *E. coli* strain DH5α [9] was used for the routine growth and maintenance of plasmids. All media for the growth of DH5α and procedures of transformation were standard [10].

The yeast *E. coli* shuttle vector pAD4 used in this study has been described previously [11]. The authentic killer preprotoxin gene sequence was obtained by Dr. A. Meškauskas [12] and recloned into the *Sal*I site of pAD4. The resulting plasmid, pYEX12-D, conferred the killer and immunity phenotypes on the α'1 yeast strain [11]. Site-directed mutagenesis was carried out by using this plasmid. General methods for DNA manipulations involving ligation, restriction digestion, T4 DNA polymerase treatment, dephosphorylation, electrophoresis, extraction of DNA from agarose gel were performed essentially as described in [10]. To ensure that the correct construction had been achieved, all plasmids were checked by restriction mapping. Both orientations (direct – D and reverse – R) of the killer preprotoxin gene were picked out in cases of all constructions.

## RESULTS AND DISCUSSION

Our initial approach was to study the phenotypes of a set of mutations through the K2 killer preprotoxin gene. For the construction of pBam-D and pBam-R, 1.2 kb *Bam*HI-*Bam*HI restriction fragment containing preprotoxin gene lacking the first in-phase start codon was removed from pYEX12-D, purified by electrophoresis, extracted from agarose gel and made blunt-ended with T4 DNA polymerase. pAD4 was linearized with *Sal*I and treated with the T4 DNA polymerase and Calf intestine alkaline phosphatase prior to ligation. Transformants in the sensitive α'1 strain showed lowered the secretion of a toxin (Figure, line 5) in comparison with wild-type levels (plasmid pYEX12-D, Fig., 3 line) and had partially inactivated the immunity (K2 type killers formed small killing zones in plate tests). The stability of pBam-D was 89.55 ± 0.63%, while wild-type pYEX12-D showed a stability of 92.00 ± 0.85%. The unexpected result was production of toxin (though at low levels) by transformants with an inverted orientation of preprotoxin gene without start codon (plasmid pBam-R, Figure, line 6). These transformants failed to display the immunity, as K2 killers formed large and clear zones on the lawn of transformants, and transformants showed small zones themselves. The stability of pBam-R was 75.63 ± 1.60% and we observed cases of independent elimination of altered preprotoxin gene (9.05 ± 1.07%), while the rest part of plasmid was maintained in the cell. We suppose that such a phenomenon and the low stability of pBam-R could be determined by a suicide phenotype of transformants. On the basis of these results we constructed the plasmid pYEX12-R. pYEX12-R was derived from pYEX12-D by recloning the native killer preprotoxin gene to *Sal*I site of pAD4 vector in an inverted orientation with respect to the ADH1 promoter. Surprisingly, transformants formed wild-type size killing zones on the lawn of α'1 (Figure, line 4), but were sensitive to K1 and K2 killers and their own toxin. The stability of pYEX12-R was 73.91 ± 1.15%. These results showed that the presence of ADH1 promoter was indispensable to assure the normal immunity, but secretion of a toxin was possible in its absence. So, we deleted with *Bam*HI flanking sequences of wild-type K2 cDNA in plasmid pYEX12-D (ADH1 promoter in 5'-end and sequencing primer in 3'-end) and constructed plasmids pADHΔ-D and pADHΔ-R. Plate tests indicated that the production of the toxin was slightly lowered (Figure, line 7 – pADHΔ-D; line 8 – pADHΔ-R) and the transformants did not display immunity. The stability of pADHΔ-D was 86.54 ± 1.12% and of pADHΔ-R 74.59 ± 1.96%. pBamADHΔ-D and pBamADHΔ-R were obtained by ligation the 1.2 kb *Bam*HI-*Bam*HI

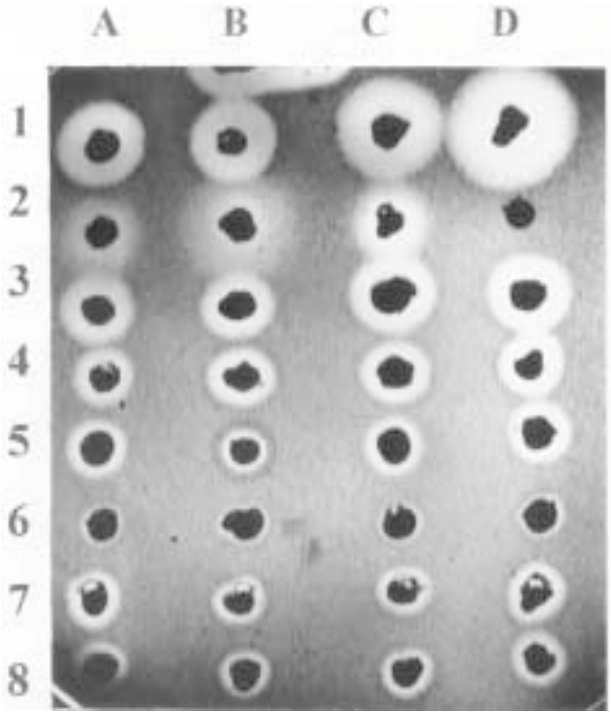


Figure. Effect of mutations on K2 killer activity. Similar amounts of test cultures were spotted on a sensitive strain α'1 seeded plate. line 1, A, B – wild-type (wt) M1 dsRNA strains; 1 line, C, D – wt M2 dsRNA strains; line 2, A, B, C – wt M28 dsRNA strains; line 2, D – sensitive strain α'1; line 3 – wt K2 cDNA containing strain α'1[pYEX12-D]; line 4 – wt K2 cDNA in an inverted orientation towards the ADH1 promoter, α'1[pYEX12-R]; line 5 – K2 cDNA lacking the first start codon, α'1[pBam-D]; line 6 – reverse orientation of K2 cDNA without start codon, α'1[pBam-R]; line 7 – wt K2 cDNA in plasmid without ADH1 promoter, α'1[pADHΔ-D]; line 8 – reverse orientation of wt K2 cDNA in plasmid pADHΔ, α'1[pADHΔ-R]

DNA fragment carrying a killer preprotoxin gene without the first start codon with 6 kb *Bam*HI-*Bam*HI fragment of pYEX12-D (vector without ADH1 promoter and sequencing primer). These mutations resulted in a low secretion of toxin and clearly led to defective immunity (transformants formed killing zones like those of transformants with plasmid pBam-D (Figure, line 5)). Thus, although the plasmid pYEX12-D with the cDNA insert controlled by the ADH1 promoter produced toxin and was immune to its action, the plasmids containing cDNA in an inverted orientation or lacking ADH1 promoter produced lower levels of toxin, and the transformants were sensitive. A similar situation has been observed in transformants with a killer preprotoxin gene without the first start codon. These findings enabled us to suppose that the leader sequence of preprotoxin is important in expression of immunity; production of killer toxin is not totally dependent on ADH1 promoter and may occur in its absence; ADH1 promoter may influence the formation of immunity.

**References**


1. Bussey H, Boone C, Zhu H et al. Experientia 1990; 46: 193–200.
2. Wickner RB. Annu Rev Microbiol 1992; 46: 347–75.
3. Magliani W, Conti S, Gerloni M et al. Clin Microbiol Rev 1997; 10 (3): 369–400.
4. Dignard D, Whiteway M, Germain D et al. Mol Gen Genet 1991; 227: 127–36.
5. Читавичюс Д, Инге-Вечтомов СГ. Генетика 1972; 1: 95–102.
6. Ito H, Fukuda Y, Murata K et al. J Bacteriol 1983; 153: 163–8.
7. Наумова ТИ, Наумов ГИ. Генетика 1973; 9 (4): 85–90.
8. Sherman F, Fink GR, Hiks JB. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, New York, 1986.
9. Woodcock DM, Crowther PJ, Doherty J et al. Nucleic Acids Res 1989; 17 (9): 3469–78.
10. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
11. Meškauskas A, Čitavičius D. Gene 1992; 111: 135–9.
12. Meškauskas A. Nucleic Acids Ress. 1990; 18 (22): 6720.


**G. Gulbinienė, T. Jokantaitė, V. Melvydas**

**MIELIŲ K2 KILERINIO PREPROTOKSINO GENO MUTACIJŲ ĮTAKA TRANSFORMANTŲ FENOTIPUI**


S a n t r a u k a

Mielių kilerinio preprotoksino genas koduoja ir kilerinį toksiną, ir atsparumą jam. Mes ištyrėme geno lyderinės sekos mutacijų įtaką transformantų fenotipui. Taip pat tirta ADH1 promotoriaus įtaka geno ekspresijai. Nustatyta, kad geno lyderinė sritis yra svarbi imuniteto susidarymui, ir mutacijos joje lemia savižudišką transformantų fenotipą. Kilerinio fenotipo pasireiškimas nėra visiškai priklausomas nuo reguliuojančių sekų, bet jos yra svarbios imuniteto sudarymui: pašalinus ADH1 promotorių, transformantai yra jautrūs visų tipų kilerių poveikiui, tačiau patys sintetina kilerinį toksiną.