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A novel X factor secreted by yeast inhibits *Saccharomyces cerevisiae* K1, K2 and K28 killer toxins

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A new killer phenotype possessing the Kx strain was isolated from Lithuanian apple wine yeast population. It has been determined to belong to *Saccharomyces cerevisiae* species. The yeast was investigated by killing and immunity tests, evaluation of toxin activity and dsRNA analysis. Activity of Kx yeasts against *S. cerevisiae* K1, K2 and K28 killers and at the same time the lack of immunity as well as detection of a novel 2.5 kb M dsRNA fragment suggest presence of a new killer toxin. In this work, the Kx strain was shown to inhibit all known *S. cerevisiae* toxins. A detailed genetic and biochemical analysis is in progress to prove this phenomenon as being determined by a novel Kx toxin.

Key words: *Saccharomyces cerevisiae*, dsRNA, toxin, immunity, wine yeast

INTRODUCTION

The killer phenotype, i. e. toxin production and functional immunity, is fairly frequent among yeast and is found in natural yeasts isolates as well as in laboratory yeast strain collections [1]. In particular, for yeast strains living in their natural habitat, toxin production can confer a marked advantage in the competition with sensitive yeast strains for limited available nutrients [2]. After the discovery of killer phenomenon in *Saccharomyces*, it soon became evident that killer strains can also be found among many other yeast genera: *Debaryomyces, Pichia, Kluyveromyces, Candida, Cryptococcus, Ustilago, Williopsis* [3]. The genetic basis for killer phenotype expression can be associated with the presence of dsRNA (*S. cerevisiae, H. uvarum, U. maydis*), dsDNA (*K. lactis, P. acociae, P. inositovora*) or nuclear genes (*P. farinosa, W. makii*) [3–5]. Beside toxin-secreting strains, a significant number of non-killer yeasts can be isolated. They have lost their ability to produce various killer toxins but nevertheless retain immunity [4].

S. cerevisiae killer phenotype (K1, K2 and K28) is associated with presence of cytoplasmically inherited different size M dsRNA (satellite virus) coexisting with L-A dsRNA (helper virus) [6]. Different killer types are clearly distinguished by a lack of cross-immunity, toxin properties, and killing mechanism [7–9]. Members of all three groups are capable of killing non-killer yeasts as well as killer yeasts of the opposite killer class, remaining immune to their own toxin and to the strains belonging to the same killer group. The killer toxins K1 and K2 bind to β-1,6-D glucans in the cell wall and disrupt membrane function [10–12], whereas the killer toxin K28 binds to α-1,3-linked mannoprotein and causes early inhibition of DNA synthesis [3]. The mechanism for immunity is explained differently: an incompletely processed toxin travels via the secretory pathway to the external membrane surface and competes with the secreted toxin for binding [13], or the internal toxin inhibits TOK1 chanels and suppresses activation by the external toxin [14].

Killer activity has been detected in yeasts isolated from established vineyards and wineries in various regions of the world. This occurrence has prompted interest in preventing the spoilage during wine production, improving fermentation and product quality [15–17].

In the previous work, we reported results of a study of killer strains isolated from spontaneous fruit and berry wine fermentation [18]. According to the tests of the killer characteristics and immunity as well as dsRNA analysis, the isolated strains were divided into several groups. In the present work, we have identified a new killer strain producing a barely detectable amount of toxin capable of killing all three previously known types of killer yeasts, but preserving resistance to its own toxin. The new killer strain is able to inhibit K1, K2 and K28 toxin action on sensitive yeasts, at the same time remaining sensitive to all these toxins.

MATERIALS AND METHODS

Strains and media

The test wine yeast strain Kx was obtained from the cooperative society "Vaisių sultys", and the pure culture was isolated by repeated cultivation.

The *S. cerevisiae* strain α'1 (MATα leu2-2 (KIL-0)), sensitive to all killers [19], was used for testing killer toxin activity. The following yeast *S. cerevisiae* strains were used for immunity tests throughout this study: K7 (MATα arg9 (KIL-K1) [20],

Rom-K100 (wild type, HM/HM (KIL-K2) [21], M437 (wild type, HM/HM (KIL-K2) [22] and MS300 (MATα leu2 ura3-52 (KIL-K28) [23].

All yeast cultures were grown in YEPD (1% yeast extract, 2% peptone and 2% glucose) or in buffered methylene blue (MB) (YEPD adjusted to the required pH using 200 mM citrate-phosphate buffer, with 0.03% methylene blue) media depending on experiment needs.

The identification of yeast strains was done at the Microbiological Laboratory of Lithuanian Public Health Centre. An automatised mini AP I 20 CAUX system for clinical yeast identification was used applying methods and reagents of Merieux Foundation (France).

Killer assay and immunity tests

Killer activity was measured in a plate and well agar diffusion assay. Briefly, for plate test the test strains were spotted onto MB agar plates (pH range from 3.6 to 5.6) seeded with a layer of the sensitive strain α'1. After incubating the plates for 3–5 days at 26 °C, clear zones of growth inhibition surrounding the killer cells (indication of toxicity) were measured. Killer activity was interpreted as the size of the growth inhibition zone.

For measuring killer activity in a well format, test killer toxins were prepared by growing yeast strains in a liquid MB medium without methylene blue at different pH values for 96 h at 20 °C. Yeast cells were isolated by centrifugation at 3000 × g for 10 min at 4 °C and the supernatant was filtered through a sterile 0.22-μm polyvinyliden fluoride membrane. The activity of killer toxins was tested using a lysis zone assay by pipeting 0.1 ml of the resulting supernatant into wells (10 mm in diameter) cut into the agar. The diameter of the growth-free zone around the well is proportional to the logarithm of the killer toxin activity. Killer activity was expressed in arbitrary units and calculated according to the formula $D = (5 \times \log A) \times 10$, where D is a growth-free zone in mm and A is toxin activity (U / ml) [23].

The sensitivity / immunity tests were performed by patching colonies of killer strains onto the MB plates with an overlay of the yeast strain of interest (approximately 10^6 cells per plate). In this assay, yeast strains incapable of conferring functional immunity to K1, K2 or K28 killers show a sensitive phenotype, resulting in a cell-free growth inhibition zone, whereas a resistant yeast does not.

dsRNA preparation

dsRNA were prepared from yeasts grown until the late logarithmic / early stationary phase at 30 °C in YEPD medium according to the procedure described by Fried and Fink [24] with the following modifications. In order to quantitatively remove proteins from the nucleic acids, a crude cell extract was incubated for 30 min in 50 mM Tris-H_2SO_4 (pH 9.3) containing 2.5% 2-mercaptoethanol. Then it was transferred to a solution containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM Na_2EDTA, 0.2% sodium dodecyl sulphate, gently shacked for 1.5 h, and an equal volume of bidistilled phenol was added. After 10 min of centrifugation at 5000× g, the aqueous phase was mixed with an equal volume of chloroform. Nucleic acids were recovered from the aqueous phase by precipitation with 2.5 volumes of 96% ethanol and stored at –20 °C. After 15 min of centrifugation at 16 000 × g, the pellet was dissolved in 400 μl of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM Na_2EDTA). The resulting dsRNA was fractionated by electrophoresis on 1% agarose gels and stained with ethidium bromide (0.5 μg / ml).

RESULTS AND DISCUSSION

Apple, cranberry, chokeberry and Lithuanian red grape wine yeast populations were used for the determination of killer yeast occurrence. We have recently reported that occurrence of killer yeast in wine yeast population, depending on the sort and quality of material, ranged from 0.09 ± 0.04 to 67.50 ± 0.93% [18]. Major groups of identified wine killer yeast belong to the genera *Saccharomyces* and *Candida*. All strains under investigation were tested for differences in both their killing and immunity cross-reactions. These distinctions reflect differences in both production of killer toxin and immunity systems and allow division of the strains into different groups [18, 25].

In the present work, we focus on a non-typical killer strain (Kx) identified from apple wine yeast population. According to results of identification, these yeast cells belong to *Saccharomyces cerevisiae* species. Well characterized *S. cerevisiae* K1, K2 and K28 killer strains all are shown to be able to kill non-killer yeasts as well as yeast of the opposite killer type, while the producing yeast remains immune to its own toxin and to that produced by strains of the same killer type [4]. It is important to note that the yeast strain Kx shows a killer phenotype, giving small but clear lysis zones not only on the sensitive strain α'1, but also on the lawn of K1, K2 and K28 type killer strains (Fig. 1). The observed pH optimum for the Kx strain ranged between different acidic pH values from 4.0 to 5.2. Killer properties were compared with known *S. cerevisiae* killer types which show different pH optimums: from 4.6 to 4.8 for K1 toxin [7], 4.0–4.3 for K2 [8], 5.0–5.8 for K28 [9].

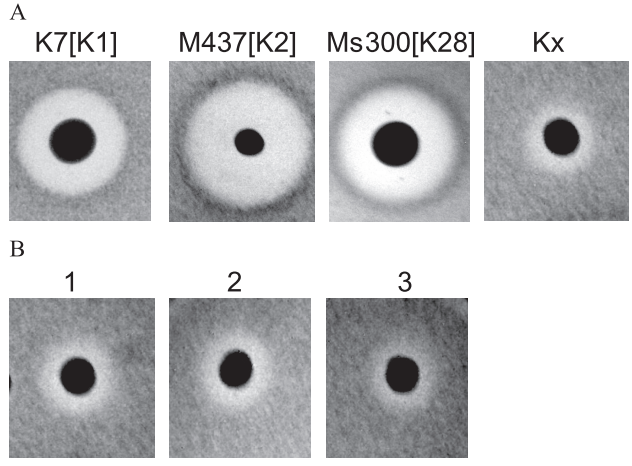

Fig. 1. Phenotype of Kx and standard killer strains of yeast *S. cerevisiae*
(A) Kx strain expressing functional toxin as well as standard killer strains K7 [K1], M437 [K2], MS300 [K28] spotted onto methylene blue agar (MB) seeded with a lawn of a sensitive non-killer strain α'1. After 3 days of incubation at 26 °C, clear zones of growth inhibition around the spots were observed (indication of toxicity). (B) Kx strain was spotted onto MB plates seeded with K7 [K1] (lane 1), M437 [K2] (lane 2), MS300 [K28] (lane 3) strains. Clear zones of growth inhibition surrounding the killer cells show Kx toxin activity against K1, K2 and K28 killers

To estimate the toxin secretion level, the Kx strain was grown in a liquid MB medium without methylene blue (pH 4.3) for 96 h (see Materials and Methods). The supernatant of the test strain expressed a barely detectable amount of toxin – 15.3 U/ml. For comparison, K2 killer expressing strain M437 at pH optimum 4.3 produces about 125.9 U/ml of toxin, and another wild type strain, Rom-K100, 79.4 U/ml.

Immunity tests showed that the Kx strain was fully sensitive to all killer types, being resistant only to their own toxin. Activity against all *S. cerevisiae* killer types and the loss of immunity suggest presence of a new killer protein.

Attention should be focused on the non-typical inhibition of the killing activity. In particular, the K1, K2 and K28 killers spotted on plates with the sensitive strain α'1 showed large zones of growth inhibition (Fig. 1). On the other hand, the Kx strain demonstrates a weak killer phenotype (Fig. 1). Surprisingly, we observed that the standard strong toxin diffusion in the medium and its killing ability can be stopped by the weak Kx strain. As is shown in Fig. 2A, the form of lysis zones was distorted and the action of K1, K2 and K28 type toxins was inhibited. The inhibitory effect depended on the distance from the point of strain seeding (data not shown). This inhibitor of toxins, secreted by the Kx strain, was called the X factor. In case of standard killer toxin interactions, this phenomenon has never been observed (Fig. 2B). All toxins diffused in the medium and showed a non-altered killer activity not dependent on toxin dose.

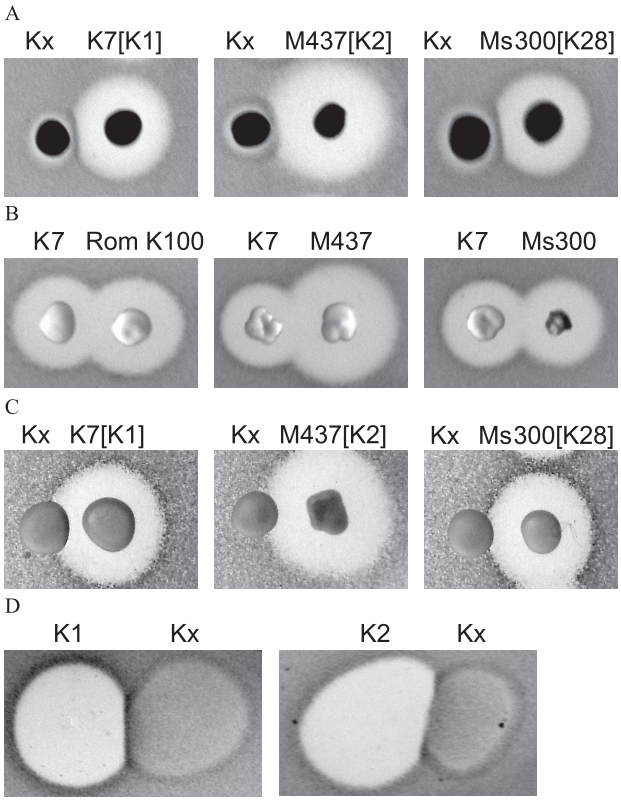

Fig. 2. Inhibition of *S. cerevisiae* K1, K2 and K28 toxins by Kx strain secreted X factor. (A) Kx and standard killer strains K7 [K1], M437 [K2], MS300 [K28] were spotted onto sensitive α'1 strain lawn and grown for 3 days at 26 °C. The Kx secreted X factor inhibits the killing ability of K1, K2 and K28 toxins and affecting form of lysis zones. (B) Effects of standard killer toxins K7 (K1 type of killer), Rom-K100 and M437 (K2 type of killer), MS300 (K28 type of killer) on sensitive strain α'1 does not depend on the distance from the point of strain seeding and toxin interaction. (C) The Kx strain after growing at 37 °C loses M dsRNA fragment, and this causes disappearance of killing ability as well as toxin inhibitory effect. (D) The Kx strain secreted X factor possesses weak killer features and ability to inhibit K1 and K2 toxins

To find out whether differences in killing and immunity properties of the Kx strain and the observed toxin inhibitory effect may be associated with the secreted toxin, additional experiments were carried out. At first, total dsRNA was extracted from the Kx strain as well as from the standard killer strains K7 (type K1), M437 (type K2), and MS300 (type K28). The extracted material was resolved in 1% agarose gel. The killer phenotype of *S. cerevisiae* strains is known to be based upon the presence of two major dsRNA segments – L and M. L dsRNA is required for maintenance and replication of M dsRNA and has a similar mobility, therefore it could not be distinguished from the L species of the representative *S. cerevisiae* K1, K2 and K28 killer strains. Killer phenotype is associated with the presence of different size M dsRNA [6]. We demonstrated that the standard killer strains K7, M437 and MS300 contained 2.0 kb, 1.8 kb and 2.2 kb M dsRNAs respectively as well as a similar size L dsRNA (about 4.3 kb) (Fig. 3). Electrophoretical analysis of dsRNA obtained from the Kx strain and a comparison with all known killer types allowed to detect a typical L dsRNA segment and M dsRNA completely different in size– 2.5 kb. This finding enabled us to suspect that the killer strain Kx with altered killer and immunity properties produces a new type of toxin.

About 3% to 5% of Kx strain clones after growing at 37 °C in YEPD medium didn't show any killing properties and at the same time the toxin inhibitory effect disappeared (Fig. 2C). This phenomenon is expected to be related to the instability of the M dsRNA segment.

Also, the X factor secreted by the Kx strain can be efficiently concentrated using Millipore (Amicon) PM10 ultrafiltration membranes. The supernatant fraction passed through the membrane didn't show any killer activity or inhibitory effect, while the concentrated fraction possessed the newly observed properties (Fig. 2D). Disappearance of the inhibitory effect together with the loss of killer abilities by the Kx strain suggest the X factor to be the same weak killer toxin possibly encoded by M dsRNA fraction. Only cloning of this gene and analysis of

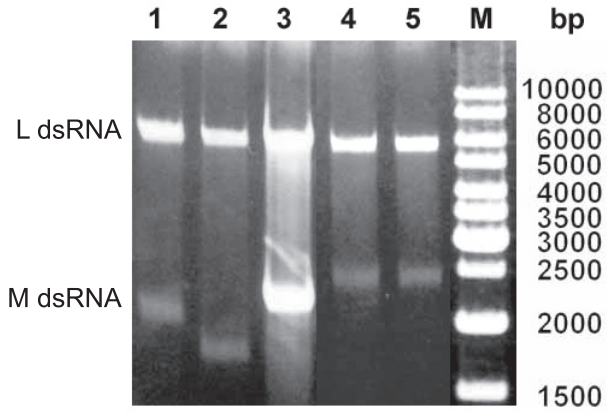

Fig. 3. Agarose gel electrophoresis of dsRNA extracts from yeast.
Lane 1, strain K7 [K1]; lane 2, strain M437 [K2]; lane 3, strain MS300 [K28]; lanes 4 and 5, strain Kx; M, size marker (Gene Ruler™ DNA ladder Mix, Fermentas)

the secreted protein can confirm or decline the hypothesis of the Kx strain toxin and the X factor to be the same product. A high competent ability can be characteristic of such a strain, providing significant advantages among wine yeasts.

Research on killer yeasts for industrial application is relatively new. The more strains from nature are screened for their range of toxin activity, the better their complex genetic, regulating mechanisms, compatibility, and level of toxin production will be understood. This increasing knowledge will allow the selection or construction of wine, beer and other industrial yeasts with killer activities targeted against a broad range of undesirable wild-type yeasts.

CONCLUSIONS

A new killer strain, Kx, was obtained from killer yeasts isolated from spontaneous fruit and berry wine fermentations. It has been characterized according to results of killing and immunity cross-reactions, toxin activity tests, dsRNA analysis. This strain was shown to secrete an X factor (most likely a killer toxin), which is capable of inhibiting all known *S. cerevisiae* toxins. This killer strain exhibits industrially significant properties that may be effective in suppressing wild yeast strains during fermentation.

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MIELIŲ SEKRETUOJAMAS X FAKTORIUS INHIBUOJA *Saccharomyces cerevisiae* K1, K2 IR K28 KILERINIUS TOKSINUS

S a n t r a u k a

Analizuojant obuolių, spanguolių, šermukšnių ir vynuogių spontaninius raugus aptiktas naujomis kilerinėmis savybėmis pasižymintis *S. cerevisiae* kamienas Kx. Nustatyta, kad Kx pasižymi plačiu kileriškumo spektru – sugeba formuoti lizės zonas ne tik ant jautraus α'1, bet ir ant K1, K2 ir K28 kilerinių tipų mielių gazonų. Tuo tarpu imuniteto testas rodo, kad ląstelės išlieka jautrios visų tipų toksinų poveikiui. Atlikus RNR elektroforetinę analizę nustatyta, kad kilerines savybes lemia 2,5kb dvigrandėje RNR koduojamas baltymas. Biocheminiai tyrimai rodo, kad naujai sekretuojamas toksinas veikia plačiame pH spektre (nuo 4,0 iki 5,2), tačiau aktyvumas yra žemas (15,3 U/ml). Nustatytas unikalus naujai atrasto kamieno bruožas – sugebėjimas stabdyti visų tipų kilerių veikimą. Remiantis tyrimų duomenimis, šį fenomeną sąlygojantis baltymas sietinas su sekretuojamu kileriniu toksinu ir yra pavadintas X faktoriumi.