A Novel *Saccharomyces cerevisiae* Killer Strain Secreting the X Factor Related to Killer Activity and Inhibition of *S. cerevisiae* K1, K2 and K28 Killer Toxins

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Abstract It was determined that Kx strains secrete an X factor which can inhibit all known *Saccharomyces cerevisiae* killer toxins (K1, K2, K28) and some toxins of other yeast species—the phenomenon not yet described in the scientific literature. It was shown that Kx type yeast strains possess a killer phenotype producing small but clear lysis zones not only on the sensitive strain α '1 but also on the lawn of *S. cerevisiae* K1, K2 and K28 type killer strains at temperatures between 20 and 30 °C. The pH at which killer/antikiller effect of Kx strain reaches its maximum is about 5.0–5.2. The Kx yeast were identified as to belong to *S. cerevisiae* species. Another newly identified *S. cerevisiae* killer strain N1 has killer activity but shows no antikiller properties against standard K1, K2 and K28 killer toxins. The genetic basis for Kx killer/antikiller phenotype was associated with the presence of M-dsRNA which is bigger than M-dsRNA of standard *S. cerevisiae* K1, K2, K28 type killer strains. Killer and antikiller features should be encoded by dsRNA. The phenomenon of antikiller (inhibition) properties was observed against some

killer toxins of other yeast species. The molecular weight of newly identified killer toxins which produces Kx type strains might be about 45 kDa.

Keywords Killer/antikiller activity · Interaction · dsRNA · *Saccharomyces cerevisiae* · X factor · Standard toxin inhibition

Introduction

The intensive studies of killer systems and search for strains with new killer characteristics in *Saccharomyces cerevisiae* promotes the importance of the species. This organism is one of the most widely used research objects in scientific laboratories and an important industrial microorganism, especially in wine fermentation. They are also widely used in food industry, pharmacy, medicine, and some other biotechnology fields [1, 2].

Yeasts, including yeasts with killer properties, are widely spread in nature, laboratory collections, food, beer and wine fermentation processes and clinical isolates. The killer phenomenon was first described in *S. cerevisiae* by Bevan and Makower in 1963, when killer strains were extracted from the infection in brewery [1, 3]. Killer yeast strains are toxin secreting fungi, that are immune, however, to their own toxin or to toxins produced by strains of the same killer type. These exotoxins are proteins or glycoproteins lethal to sensitive yeast, fungi or bacteria strains [2]. The killer yeast strains are found in many yeast genera such as *Saccharomyces*, *Candida*, *Cystoflbasidium*, *Cryptococcus*, *Debaromyces*, *Filobasidium*, *Hansenula*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Sporodiobolus*, *Williopsis*, *Zygosaccharomyces*, *Mrakia frigida* [4, 5]. Genetic

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determinants of killer toxins can be cytoplasmic dsRNA viruses, linear DNA plasmids, and chromosomally encoded [1]. Killer yeasts are spread in different climatic zones from northern to southern regions. The psychrotolerant yeast *M. frigida* 2E00797, isolated from sea sediments in Antarctica, was found to be able to produce killer toxin against *Metschnikowia bicuspidata* WCY yeasts, *Candida tropicalis* and *Candida albicans* [5, 6]. Chinese scientists isolated and characterized extracellular toxin of the marine yeast strain *Williopsis saturnus* WC91-2 which could kill many sensitive yeast strains, including the pathogenic ones [7]. The yeast *Kluyveromyces siamensis* HN12-1 isolated from mangrove ecosystem was found to be able to produce killer toxin against the pathogenic yeast (*M. bicuspidata* WCY) in crab [8], and a novel killer toxin produced by the marine-derived yeast *Wickerhamomyces anomalus* YF07b had killing activity against *Yarrowia lipolytica*, *S. cerevisiae*, *Metschnikowia bicuspidata* WCY, *C. tropicalis*, *C. albicans* and *K. aestuarii* [9]. Ecological studies indicate that killer activity is a mechanism of interference competition with the production of a toxic compound from one yeast excluding other yeasts from its habitat [10]. A killer toxin (Kbarr-1) produced by wine yeast *Torulaspora delbrueckii* strains was evaluated as a new killer toxin. It was found that this killer toxin is encoded by dsRNA and is able to kill all the previously known *S. cerevisiae* killer strains, in addition to other *non-Saccharomyces* yeasts [11].

Saccharomyces cerevisiae killer toxins are best characterized. They have been grouped into three types: K1, K2 and K28 based on their genetic determinants, toxin properties, killing profiles and entry pathways into cell. These toxins kill not only sensitive cells of the same or related yeast species, but they can kill nonkiller yeasts as well as yeasts belonging to the other genera [12, 13].

When studying the prevalence of *S. cerevisiae* killer yeasts in natural environment, the rate at which K1 and K2 killer yeasts are distributed was determined [14–18]. However, there was no data about yeast secreting factors which would inhibit or stop *Saccharomyces* killer toxins K1, K2 or K28. Our initial results presenting first such toxin, yeast secreted X factor, which is a weak killer and inhibit K1, K2 and K28 killer toxins were published in 2007 [17]. Later, the Klus type toxin with similar properties was isolated from spontaneous fermentations of grapes from vineyards in Spain [19]. The Klus phenotype yeast strains killed all the previously known *S. cerevisiae* killer strains in addition to other yeast species (including *Kluyveromyces lactis* and *C. albicans*). These findings suggest that it is possible to find new killer toxins (also in *S. cerevisiae*), including those possessing previously not described features. Recently two killer toxins from *S. cerevisiae* strains (Cf8 and M12 strains) isolated from wineries in Argentina were partially characterized. It was

found that genetic determinants of the killer phenotype could be chromosomally encoded. The newly found killers were able to kill *Brettanomyces bruxellensis*, *Dekkera anomala*, *Pichia membranifaciens*, so they could be useful improving winemaking and other industrial strategies [20]. Over a year ago six *S. cerevisiae* isolates extracted from the fermentation process for ethanol production were evaluated as possible biological agents against postbloom fruit drop caused by *Colletotrichum acutatum*, because all of these isolates produced antifungal compounds and exhibited killer activity against *S. cerevisiae* NCYC 1006 [21].

The aim of this work was to search and analyse the new yeast killer strains in spontaneous fermentations of fruits and berries gathered in different regions of Lithuania.

Materials and Methods

Yeast Strains and Media

The *S. cerevisiae* strain α' 1 [MAT α leu2-2 (KIL-0)] sensitive to all killers was used for testing killer toxin activity. The following *S. cerevisiae* reference strains were used for immunity tests throughout this study: K7 (MAT α arg9 (KIL-K1), Rom-K100 (wild type, HM/HM (KIL-K2), M437 (wild type, HM/HM (KIL-K2) and MS300 (MAT α leu2 ura3-52 (KIL-K28). The yeast strain Kx was isolated from fermentations of apples in the cooperative society “Vaisių sultys”, and the pure culture? Was isolated by repeated cultivation [22].

The yeast strains with killer activity were obtained from the spontaneous fermentations of fruits and berries gathered in different regions of Lithuania in the period of 2004–2013. Samples of spontaneous fermentations were prepared by grinding fruits and berries diluted with a small amount of water and kept for about 1 month at room temperature. Yeast isolation was done by the spread plate method on YEPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) and incubation from 3 to 5 days at 25 °C. Selected colonies were streaked on YEPD agar to obtain pure cultures and used in assays for the killer phenotype and immunity tests. The tested yeast strains were spotted on the methylene blue [19] agar plates containing YEPD adjusted to the required pH using citrate–phosphate buffer depending on experiment needs [17].

Yeast Strains Identification

The primary identification was performed by an automated mini API 20 CAUX system (bioMérieux-Vitek, Hazelwood, Mo.) for clinical yeast identification. The final confirmation was performed by phylogenetic analysis of the nucleotide sequences of NTS1/IGS1 region [23, 24].

Assay of Killer Phenotypes

Killer activity was determined by the well-test method on agar plates. The plates were prepared with agar of two layers: the basis and the layer with a sensitive culture lawn. For the search of new killer strains, agar media with different basis of thickness (ratio of layers 1:1; 2:1; 3:1 and 4:1) was used. Density of sensitive cells in the lawn did not exceed 10^6 cells/mL.

Killer activity was measured on an agar plate and well diffusion assay. Briefly, the test strains were spotted onto MB agar plates (pH range from 3.6 to 5.6) seeded with a layer of sensitive strain $\alpha/1$. After incubating the plates for 3–5 days at 25 °C, clear zones of growth inhibition surrounding the killer cells (indication of toxicity) were measured. Each experiment was replicated not less than 3 times. Killer activity was interpreted as the size of the growth of the inhibition zone. For measuring killer activity in a well format, the test killer toxins were prepared by growing yeast strains in a liquid medium.

Analysis of inhibition with different killers was performed in two ways: applying Kx onto the center with the test killer strains residing on the sides and vice versa.

Isolation of Yeast Killer Toxins and Protein Electrophoresis

The killer toxins producing strain was grown for 3–4 days in modified liquid MB medium. (pH 5.2 for Kx and pH 4.4 for strain N1) at 18–20 °C following centrifugation at $5000 \times g$ for 10 min at 4 °C. The supernatant was filtered through PVDF membrane (pore size 0.22 μ m) and concentrated at 4 °C temperature using Millipore NMWL PM-10 membrane.

After each concentration, the activity and interaction of the killer toxin was tested. For primary testing, filtered or concentrated supernatant samples were dropped (10, 25, 50 μ L) on plates with $\alpha/1$ strain, and the formation of lysis zones was observed after 4 days of the incubation at 18–20 °C. Concentrated proteins were analysed using SDS-PAGE method [25].

Isolation and Electrophoresis of Double-Stranded RNA

dsRNA was prepared from yeasts grown to the late logarithmic/early stationary phase at 30 °C in YEPD medium according to the procedure described by Fried and Fink [26] with some modifications [17]. The resulting dsRNA was fractionated by electrophoresis on 1 % agarose gel and stained with ethidium bromide.

Extraction of Yeast DNA

Yeasts DNA was extracted after 48 h of the growth on YEPD agar. Yeast genomic DNA was extracted by Thermo Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's recommendations.

Polymerase Chain Reaction (PCR) and DNA Sequencing

NTS1/IGS1 region of rRNA genes was amplified with primers P170 (5'GAACCATAGCAGGCTGGCAACGGTGCTCTTAGC) (complimentary to 5 s rDNA) and P172 (5'CCATATCTACCAGAAAGCACCGTTTCCCGTCC) (complimentary to 25 s rDNA) with the following PCR profile 95 °C for 3 min 25 cycles for 30 s, 68 °C for 1 min, and the final extension at 72 °C for 10 min. Thermo Scientific DreamTaq DNA Polymerase was used to amplify appropriate regions of yeasts DNA under the manufacturer's recommendations. DNA amplicons of about 1300 nucleotides length were cloned into the plasmid pJET1.2 using CloneJET PCR Cloning Kit (Thermo Fisher Scientific Baltics) according to the manufacturer's recommendations. Both strands of the inserted DNA were sequenced using ABI BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) using standard pJET1.2 DNA primers and analyzed with ABI 3130xl Genetic Analyzer. The comparison of the IGS1 sequences of the test strains with the whole-genome shotgun contigs (wgs) at NCBI database (<http://blast.ncbi.nlm.nih.gov>) was performed by MEGA-BLAST [27, 28]. The sequences were aligned using Muscle (YYY) program and evolutionary distances between sequences were calculated by the Tamura–Nei model using the neighbor-joining analysis method on Geneious v4.8 Tree Builder [29].

Results

Phenotypic Characteristics of Killer Yeasts

The search was done on fruits and berries gathered in different regions of Lithuania. The research was focused on most widely grown fruits and berries in Lithuania, and about 150 spontaneous fruit and berry fermentations was screened every year. The first step in every spontaneous fermentation screen was the search for strains with killer phenotype. Primary analysis was performed with sensitive *S. cerevisiae* yeast culture $\alpha/1$ on agar and methylene blue at different pH. At the same time we determined whether isolated strains have K1, K2, or K28 killer types. In some spontaneous fermentations yeast strains with differing killer activity can be found however, usually one killer type

is not dominating (Fig. 1). Kx type strains were found in such fermentations because it is the interactions between different killers.

The killing ability of newly found strains were tested comparing with standard *S. cerevisiae* killer types. Standard killer *S. cerevisiae* (K2–M437 and Rom K100, K28–MS 300, K1–DBY), *K. lactis* (M270) strains and the new Kx strain and KNX (strain screened from a mistletoe) were sprayed onto MB agar with sensitive yeast α' , pH 4.8, and analysed 4 days after incubation at 25 °C temperature. Differing killing effect of yeast strains producing *S. cerevisiae* type K1, K2 and K28 toxins, *K. lactis* toxins, and killer factors KNX and Kx was observed. The sizes of lysis zones differed from each other and mostly resembled three typical forms: *S. cerevisiae*, *K. lactis* and Kx.

The Interaction Between *S. cerevisiae* Killer Toxins K1, K2 and K28 and Killer/Antikiller Factor

The K1, K2 and K28 killer strains spotted on plates with the sensitive strain α' showed large zones of growth inhibition; the Kx strain demonstrates a weak killer phenotype. Surprisingly, we observed that the standard strong lysing action in the medium can be stopped by the Kx strain, possessing weak killer properties.

Evaluation of Killer Activity and Immunity to Killers of Strains Secreting the Inhibiting X Factor

Firstly, the pH optimum of Kx toxin activity was determined. One of the most important conditions for killer activity evaluations is the acidity of the screening medium. The killer activity occurs at acidic pH between 3 and 5.5 [13]. Strains, isolated from the spontaneous fermentations of fruits and berries, were tested for killer activity and

immunity in the same manner as standard *S. cerevisiae* K1, K2 and K28 killer strains were tested. Firstly, the pH optimum of Kx toxin activity was determined. It was determined that secreted Kx toxins are most effective at pH 5.0–5.2. In this pH interval K1, K2 and K28 strains are active as well, but the activity of the latter toxins is also observed at pH 5.6 and 4.4 when temperature is 20–30 °C. As all the strains used in this work grow well and show killer activity at 25 °C, this temperature was chosen for further experiments. Kx strain clearly killed (but producing only small lysis zones) standard *S. cerevisiae* K1, K2 and K28 killer strains at pH 5.2 and vice versa. It is dropped in dots about 10^6 cells on 10^5 lawn (different growing rates) of wild type and mutant strains, so it can be asserted, that by its function it is the new killer, comparing with *S. cerevisiae* standard strains (Fig. 2).

The Optimum pH for Production and Stability of Killer Factor

It was determined that optimum pH for the Kx yeast strain killer effect is 5.0–5.2. However, at some pH values ($\text{pH} \leq 4.4$) the killer and inhibitory effects disappear. Furthermore, at 37 °C Kx type strains lose their killer properties, a part of colonies lose the ability to inhibit the action of standard *S. cerevisiae* killer toxins. Under these conditions M-dsRNA fraction is also lost. Therefore, it can be assumed that either the killer toxin and secreted factor of inhibition may be the same toxic protein, or they both are encoded by the M fraction and their expression must be concurrent, but the latter suggestions seem unlikely.

One more killer strain of new type differing from Kx (called N1) that was found previously is used for wine production. Therefore, killer activity, similarity test of the Kx type strain and the part identification was done. Killer activity of N1 strain is very similar to Kx (about 10 %

Fig. 1 Screening for new killer strains in spontaneous fermentation. Yeast colonies after replica plating from YEPD medium to MB medium with pH 4.8 after 4 days of incubation at 25 °C. An agar seeded with the lawn of sensitive non-killer strain α' . Arrows indicate

yeast killer strains with differing activities: medium-sized lysis zones contains the toxin type K2, small lysis zones contains the Kx-type killer factor

Fig. 2 Effect of the incubation temperature and agar pH on the lysis zones produced by Kx strain. From *left to right* pH 5.2, 37 °C; pH 5.2, 25 °C; pH 4.4, 25 °C. MB medium, lawn α /1, after 6 days of incubation. The lysis zones appear after 3 days, concurrently with the inhibition effect

comparing with M437, pH optimum about 4.4. Thus, to determine the inhibition activity of N1 toxin at pH 4.8 we need to concentrate it 1000-fold, as it was made in the case of Kx toxin. The activity was not observed at pH higher than 5 and lower 3.5. N1 is not lysed by Kx killer, but it is lysed by K1, K2, K28 (results not shown). Its secreted killer toxin does not show the inhibition effect, but for other investigations it might be interesting as the a new killer strain.

PCR and DNA Sequencing

Nucleotide sequences of the rRNA gene's loci of strains Kx and N1 were used to elucidate their phylogenetic relationships. Genomic DNA was amplified with the primers P170 (5'GAACCATAGCAGGCTGGCAACGGTGCTCTTAGC) and P172 (5'CCATATCTACCAGAAAGCACCGTTTCCCGTCC) designed with reference to *Kluyveromyces marxianus* rRNA non-transcribed intergenic spacer NTS1/IGS1. These primers were successfully used for the identification of the broad range of other yeast species (unpublished observations). NTS1/IGS1 region is localized between 5S and 25S rRNR genes and the separate ribosomal RNA tandem repeats. The resulting amplicons of around 1300 bp were sequenced and 923 bp. long sequence of N1 strain and 550 bp. sequence of Kx strain were used for The National Center for Biotechnology Information (NCBI) database analysis with BLAST tool. The sets of sequences with various E-value were taken for further alignment. The sequences were aligned using Muscle [30] program and evolutionary distances between sequences were calculated by the Tamura–Nei model using the neighbor-joining analysis method on Geneious v4.8 Tree Builder [29]. Phylogenetic analysis of individual sequences are presented as bootstrap [31] consensus trees (Fig. S1). Bootstrap support was determined from 1000 replications. Algorithm used to describe phylogenetic relationships speaks in support of both strains which belong to genus *Saccharomyces*.

Establishing Genetic Determinants

In order to confirm that Kx and other similar strains are killers of the the new type, their dsRNA was extracted and analysed electroforetically. dsRNA from standard killer *S. cerevisiae* strains K7, M437 and MS300 (killer phenotype K1, K2 and K28, respectively) was extracted as a control. Comparing extracted RNA with standard strains (K1, K2 and K28) and earlier extracted Kx strains RNA, it was determined that size of dsRNA is about 2.5 kb (Fig. S2). N1 type strain does not differ from the previously found N1 strain. These do not have L and M fractions which was shown by the multiple dsRNA analysis.

Molecular Mass of the Kx Killer Protein

Secreted proteins from two Kx type killer strains (found independently) were concentrated and the electrophoresis under reducing SDS-PAGE conditions was performed (results not shown).

SDS-PAGE analysis revealed that proteins secreted by Kx type strains differ from the standard proteins produced by *S. cerevisiae* K2 killer type strain. It can be supposed that Kx type strains secrete more proteins into the medium, therefore it is impossible to discern the band of the killer protein.

The comparison of secreted protein bands derived from functional Kx type strain and the same strain that lost its killer activity would facilitate in identifying the band responsible for the toxic activity. To produce such inactive strain the cloned culture of active Kx type strain was grown for 3 days on YEPD medium at 37 °C. This method is mild, it has low potential of causing mutations or any other undesirable effects, and is widely used to remove killer toxin-encoding M-dsRNA. In order to get the pure culture, K[−] clones were cloned repeatedly. Secreted proteins of such clones were concentrated in the same way as it was done in the case of Kx (K⁺) type strains. Secreted proteins of K[−] clones were run on SDS-PAGE under reducing conditions after concentration procedure and were compared to secreted proteins of Kx strains.

Discussion

For the first time, a new killer factor X secreting *S. cerevisiae* yeast strain was isolated in our laboratory from apple wine yeast population in winery “Vaisių sultys” [17].

The analysis of yeast in spontaneous fermentations has shown the dominance of the K2 type killer yeast strains. Yeast killer strains belonging to different species and having different killer characteristics were found during each year of the investigation. About 8–10 % of all investigated yeast killer strains belonged to *Saccharomyces* genus and had K2 phenotype, only few had K1 phenotype. However, K28 killer strains are unrepresented in Lithuanian yeast population and were not isolated [18]. Our results showed that the yeast from spontaneous fermentations has a wide spectrum of killer activity: from weakly secreting killer factor to strong effect when compared to standard *S. cerevisiae* K2 type strain M437.

Twelve yeast strains with the same killer effect were found during this research (1–2 strains each year). There was no polymorphism found. Interactions of their secreted factors with standard *S. cerevisiae* K1, K2, and K28 killer toxins produced unexpected results: the form of lysis zones was of the new Kx type, i.e. the secreted factor of the newly found strains inhibited the killing effect of all standard toxins. On the contrary, the interactions between standard killer toxins has neither influence on killing effect nor results in any other detectable effect (Fig. 3a). Interacting standard *S. cerevisiae* killer strains do not show any inhibition zones (Fig. 3b).

At the temperatures used in the experiments our toxin forms very specific killing zones surrounded by growing

stimulated ring (Fig. 2). The pH of the medium needs to be maintained about 5.0–5.2. This can be explained by hypothesizing that in this case the toxin or X factor does not kill cells but irritates them, which intensifies their metabolism and reproduction. Meanwhile, the standard killer toxins start inhibiting the growth when they lack killing activity. This suggests that toxin X and hypothesized antitoxin is one and the same agent with very special characteristics. Removing M-dsRNA fraction (see chapter “Establishing Genetic Determinants”) some killer strains abolish their killer activity and the inhibition effect. When analysing this phenomenon at pH lower than 4.4, the killer and the inhibition effects disappear as well.

It was determined that isolated Kx strains dropped in dots on the rigid medium. Artificial modeling of clones with killer and inhibition features: randomly dropped spots of *S. cerevisiae* K1, K2, K28 killer strains, *K. lactis* and Kx strains on YEPD medium. Then stamps were done on MB medium. Standard *S. cerevisiae* strains with killer phenotype do not inhibit the killer toxins actions of each other. Medium MB pH 5.2 kills the standard K1, K2, K28 strains, forming small but clear zones of lysis. Before mentioned standard *S. cerevisiae* killer strains kill Kx strains, suggesting that Kx is a new killer type. The inhibition effect is sharpened by improving the methodology (Fig. 4). Figures 3 and 6 were obtained using this method.

According to the presented data, the factor determining the observed phenomenon is associated with the secreted killer toxin and we have named it X factor. Killer yeasts of this type are rare. During 10 years of the research on different samples collected in Lithuania regions (Klaipėda, Rokiškis, Vilnius, Anykščiai), seven times strains of Kx

Fig. 3 The antikiller effects of the X factor secreted by the Kx strain and the killer toxins secreted by other strains. **a** The Kx strain surrounded by M437 (left K2 type) or MS300 (right K28 type). MB medium, pH 5.2, 25 °C, lawn α 1. **b** The interaction between standard *S. cerevisiae* killer strains MS300 [K28] (above), M437 [K2] (middle) and K7 [K1] (bottom). MB medium, pH 4.8, 25 °C, lawn α 1, 4 days of incubation

Fig. 4 Modification of the method: sharpening of the inhibition effect. From left to right proportion of MB medium and lawn α 1 ~3–4:1 thickness, pH 5.2; ~1:1, pH 5.2; and ~1:1, pH 4.8. Lawn α 1. Replica of a cloned spontaneous fermentations from YEPD medium. Artificial modeling of the clones with the killer and the

inhibition features: randomly dropped spots of *S. cerevisiae* K1, K2, K28 killer strains, *K. lactis* and Kx strains on YEPD medium. Then stamps were done on MB medium. Standard *S. cerevisiae* strains with the killer phenotype do not inhibit the killer toxins' actions

type with the new killer properties were found. According to these long-termed research results, we may propose that this strain is widespread in Lithuania. It can also be expected to find these strains in other world regions and in other yeast species. It should be noted, that the strains described were found randomly in contact of *S. cerevisiae* spontaneous fermentations clones with K2 type toxins. These strains are not readily detectable because of their different conditions of actions pH, temperature and different killer yeast in nature populations. Therefore, in search of these killers all interactions among known killers as in known natural populations and in collections must be tested. Fortuity and attentiveness of investigator will be determined. In Lithuanian yeasts populations this phenomenon might be more frequent than it is found.

We present here new killer strains secreting X factor which inhibits standard *S. cerevisiae* killer toxins and some toxins produced by other species. According to the killer, immunity, inhibitory properties and phenotypic similarity, isolated strains are identical to Kx strain [18] and their killer toxin is encoded by dsRNA. *S. cerevisiae* N1 toxin is not inhibited by the concentrated Kx toxin, however the Kx toxin inhibits the K2 type killer toxin (Fig. 5).

As far as it is known, the Kx factor does not have protease activity. It is unlikely that protease activity would be encoded by dsRNA, and in this study we failed to observe any protease activity attributable to Kx yeast. Furthermore, it was observed that secreted killer toxin/antitoxin does not inhibit *K. lactis* killer toxin, secreted by *K. lactis* var. *lactis*. It was also shown that X factor can inhibit some other killer toxins (Fig. 6a).

It was also determined that Kx type strain inhibits *Pichia anomalis* and some other killer strains, which is non *Sacharomyces* spp. (not identified). In the case of *S.*

Fig. 5 The interaction between Kx and other concentrated killer toxins. In the centre Kx killer toxin, on the left the concentrated toxin of *Saccharomyces* sp. N1 strain, on the right the toxin of *S. cerevisiae* Rom-K100 [K2] strain. MB medium, pH 5.2, 25 °C, lawn α 1

cerevisiae MS300 killer strains, the inhibiting activity of the inhibition factor and killer toxin K2 differs significantly, therefore the zones of inhibition are getting smaller (Fig. 6b). The Kx inhibition zone is larger than its killer toxin lysis zone, but not larger than the stimulation zone. The interaction between the killer and antikiller strains depends on the ratio of the toxin-antitoxin secretion intensity (Fig. 6a). This observation might be explained supposing that X factor-mediated inhibition of the killer activity is the result of ongoing competition between the toxin and the antitoxin for the killer receptors-binding in the cell wall or the membrane, or the result of the direct interaction between the toxin and the antitoxin (results not shown).

In the spontaneous fermentations of fruits and berries, the strains with the killer activity similar to the strains secreting the X factor were found, but their pH optimum for the killing activity was different: as it has already been

Fig. 6 **a** The inhibition of killer toxins secreted from different yeast strains by *S. cerevisiae* 65 + ob producing X factor. Standard *S. cerevisiae* killer types: K7–K1; M437, RomK-100–K2; MS300–K28. MW270—*K. lactis* strain; medium MB, pH 5.2, 25 °C, lawn— $\alpha/1$. **b** The interaction between concentrated Kx toxin-antitoxin and K7 or MS300 spots. MB medium, pH 5.2, 25 °C, lawn $\alpha/1$. The inhibition effect depends on the distance and the ratio between lysis zones

mentioned, the optimum for Kx strains is about 5.2, whereas the optimum of these similar strains (of N1 type) is about 4.4. We compared the killer activities of these strains with each other on the sensitive lawn $\alpha/1$, on the standard *S. cerevisiae* killer strains lawns and conversely. Our results suggest that these new yeast strains might also be some new killer strains, but they do not show the inhibition activity (results not shown).

The killer activity is found not only in *Saccharomyces*, but in many other yeast genera, such as *Pichia*, *Candida*, *Kluyveromyces*, etc. Therefore, we tried to determine to which genus and species the found yeast isolates belong.

To sum up, regarding such parameters as the pH of the medium, layer thickness, density of lawns, temperatures and position (center or side), it may be proposed that clear killer effect and inhibition effect have existed in Lithuanian region for many years. It is possible, that this phenomenon will also be found in other species and in other regions.

It has already been defined that *S. cerevisiae* K1, K2 and K28 killer toxins are encoded by the genes in dsRNA molecules, which are encapsidated into virus like particles (VLP). Later, this phenomenon was investigated in other yeast genera and it was found that genetic determinants of the killer phenotype can also reside in linear DNA plasmids (for example *K. lactis* and other) or in chromosomes (some species of *Candida*, *Cryptococcus* genera and other) [12].

RNA electrophoresis revealed that the size of M fraction of the newly found Kx strains do not differ from other Kx type strains and do not coincide with the standard *S. cerevisiae* killer strain M fraction size. The sizes of M fractions of dsRNA from Kx type killer strains are about

2300–2400 bp, M fraction sizes from standard killer strains are smaller: ~1900 bp (K1 type), ~1650 bp, (K2 type), ~2200 (K28 type). According to functional manifestation (killer activity/inhibition) and multiple results of electrophoresis, this can not be said about polymorphism as in Klus [19] case. Lithuanian region is too small for polymorphic killer Kx strains to be found.

Besides, our results showed, that Kx (K^+) type strains have two fragments with molecular masses about 45 and 27 kDa absent in K^- clones. It can be supposed, that protein with molecular mass 45 kDa is the killer toxin and the factor of inhibition secreted by Kx type strain. Molecular masses of standard *S. cerevisiae* K1, K2 and K28 types killer toxins are about 19, 21.5 and 21.4 kDa respectively [1, 13]. The size of M-dsRNA may encode such protein.

This phenomenon might be useful in the area of the research of antagonistic interaction between killer yeast. It could be useful for the analysis of the general killer phenomenon in yeast, also in the fields of research of cell functions and immunity against toxic environmental factors. It is likely that similar strains might be very useful for the economic bioethanol manufacture and also in wine industry.

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