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SACCHAROMYCES CEREVISIAE KILLER TOXINS: SYNTHESIS, MECHANISMS OF ACTION AND PRACTICAL USE

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❀ Yeast *Saccharomyces cerevisiae* is a unique model for studying the molecular mechanisms of exotoxin-mediated antagonistic relationships between coexisting microorganisms. The synthesis of yeast toxins can be considered as an example of allelopathy and environmental competition. The elucidation of the role of allelopathy in the formation of microbial communities is of great interest for modern ecology. Yeast toxins are widely used in medicine, the food industry and biotechnology. The review examines the nature of exotoxins, the mechanisms of inheritance and interaction of the virus and yeast cells, as well as the prospects for their practical application.

❀ **Keywords:** cytoplasmic heredity; yeast killer toxins; allelopathy.

КИЛЛЕР-ТОКСИНЫ ДРОЖЖЕЙ *SACCHAROMYCES CEREVISIAE*: СИНТЕЗ, МЕХАНИЗМЫ ДЕЙСТВИЯ И ПРАКТИЧЕСКОЕ ИСПОЛЬЗОВАНИЕ

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❀ Дрожжи *Saccharomyces cerevisiae*, секретирующие токсины различной природы, являются уникальной моделью для изучения молекулярных механизмов антагонистических взаимоотношений сосуществующих микроорганизмов. Синтез дрожжевых токсинов можно рассматривать как пример аллелопатии и экологической конкуренции. Выяснение роли аллелопатии в формировании микробных сообществ представляет большой интерес для современной экологии. Дрожжевые токсины широко используются в медицине, пищевой промышленности и биотехнологии. В обзоре рассмотрены природа экзотоксинов, механизмы наследования и взаимодействия вируса и клеток дрожжей, а также перспективы их практического применения.

❀ **Ключевые слова:** цитоплазматическая наследственность; киллер-токсины дрожжей; аллелопатия.

BACKGROUND

The process of formation of microbial communities involves different types of organism interaction. Antagonistic relationships play an important role as they allow the species of one type to destroy or suppress the growth of another. The so-called contactless methods of struggle are frequently implemented. The secretion of various, sometimes toxic, compounds in the environment is known as allelopathy and is widely occurring in nature. Allelopathy is inherent to protozoans, sponges, filamentous fungi, and plants. The role of allelopathy in the formation of ecological microbial communities has been insufficiently studied [1], although the excretion of toxic compounds (bacteriocins and mycocins) in the environment is frequent among bacteria [2] and yeast [3, 4]. Bacteriocins and mycocins effectively fight related and remote species. They are widely spread in natural populations [1].

Yeast *Saccharomyces cerevisiae* is a convenient model for the study of exotoxin action at a molecular level. The killer yeast was detected for the first time in 1963 by Beaven and Macover [cited by 5]. It was then determined which yeasts synthesize exotoxins: the so-called killer factors. These factors interact with receptors located in the cell wall of sensitive yeasts. Exotoxins have a diverse structure and are either simply proteins or glycoproteins [6].

Killer yeasts are not sensitive to their own toxins but are sensitive to the killer factors of other killer yeasts. Killer activity can be aimed at representatives of their

own species and a wide range of eukaryotic and prokaryotic organisms [7]. Such properties of killer toxins are of great interest for biotechnology, medicine, molecular biology, and ecology. So far, killer factors have been detected in different species of yeasts; however, as they are perfectly studied in the yeast *S. cerevisiae*, this review pays great attention to toxins of this species. It examines the molecular mechanisms of the action of yeast killer factors, which synthesis is due to the RNA-containing viruses, mechanisms of immunity occurrence, and the ecological and evolutionary aspects of the phenomenon.

HOW YEASTS BECOME KILLERS AND WHETHER THE KILLER CAN BE "RE-EDUCATED"

Killer phenotypes can be stipulated by several reasons. Viruses containing double-stranded RNA (dsRNA) can be present in yeast cell cytoplasm. Besides, chromosomal genes *KHR* and *KHS* coding exotoxins with a molecular weight of 20 and 75 kDa, respectively, were detected. These exotoxins have weak killer activity in relation to *Candida glabrata* and *S. cerevisiae* [8–10]. Four groups of killer strains – K1, K2, K28, and Klus – were detected in the yeast *S. cerevisiae*, for which the killer activity is stipulated by dsRNA. Features such as peculiarities in killer toxin structure, their genetic determinants, and the presence or absence of cross-immunity were used for classification [6]. All these killer strains contain virus-like particles in cytoplasm. Viruses that ensure a "killer" phenotype are attributed to the *Totiviridae* family, in the mycovirus class. One of these viruses is the helper virus L-A; the other is the satellite virus M. Satellite dsRNA M (M1, M2, M28, and Mlus) code different toxins: K1, K2, K28, and Klus. Viruses L-A and M have their own envelope [11, 12], and the synthesis of the envelope of both viruses provides for virus L-A. Both viruses are required for the synthesis of the effective killer toxin and the formation of immunity in the host cell. Toxins can kill sensitive cells, but killer strains are resistant to their own toxin and the toxins of related groups. So-called neutral strains were detected in the study of killer strains. They did not kill sensitive cells but were resistant to the killer factor [13]. Thus, budding yeast contains virus-like particles in cytoplasm, and their functioning provides a selective advantage to the host cell in certain conditions.

Virus dsRNA never occurs in cytoplasm, and virus transfer in yeast is only possible during the budding process from the mother cell to the daughter cell or, in the case of breeding, cytoduction and sporogenesis [5, 14]. Thus, sensitive yeast can become killer only as a result of breeding with the killer strain in its natural habitat, which is a very rare event in the natural environment. However, it is rather easy to "re-educate" the killer; early works demonstrate that the loss of infectiousness of killer strains takes place under the effect of physical (high temperature and ultraviolet irradiation) and chemical (5-fluorouracil, acridine orange, and ethylmethane sulfonate, etc.) agents [15].

STRUCTURE KILLER VIRUSES AND THEIR HELPERS

The effective synthesis of virus toxins requires the joint functioning of two types of viruses: the helper virus, L-A, and the killer viruses, M1, M2, M28, and Mlus [6]. Mycovirus L-A is a self-replicating helper virus coding of a protein of capsid (Gag) and RNA-dependent RNA-polymerase (transcriptase), which are expressed as a fusion protein (GagPol). Satellite virus M contains information about the killer toxin and uses the proteins of L-A capsid and polymerase GagPol. Consequently, the presence of the L-A virus is crucial for the maintenance and replication of both mycoviruses, whereas virus M is only responsible for the development of the killer phenotype and its autoimmunity [16].

L-A viruses are isometric particles with a diameter of 39 nm; they contain a genome consisting of dsRNA and a complete nucleotide sequence of which is determined and amounts to 4579 bp. [17]. The structure of capsid has pores, through which nucleotides penetrate and the mRNA virus is released. A mature virus particle has one molecule of dsRNA and transcriptase connected to it. The process of the synthesis of the (+) RNA chain is discussed in detail in the review [6]. During *in vitro* experiments, it was demonstrated that transcriptase synthesizes the (+) RNA chain with unpaired adenin residue at the 3'-terminus. In the infected cell, the (+) chain is released from the virus particle and serves as the mRNA for the translation of virus capsid proteins on ribosomes in cytoplasm; then, the (+) RNA chain is packed in subvirus particles, in which it is transcribed and forms a (–) RNA chain forming dsRNA. Transcription of the dsRNA genome takes place inside the virion; thus, the dsRNA of the virus genome is never exposed to the cytoplasm.

The RNA of the L-A virus (4579 bp) contains two open reading frames (ORF), a virus binding site, and inner enhancer of replication. 5'-ORF (ORF1; 2043 n.) codes the main protein of the envelope (Gag; 76 kDa), whereas 3'-ORF (ORF2) codes the polymerase (Pol). ORF2 is overlapped with ORF1 for 130 nucleotides and is synthesized only as 180 kDa GagPol (fusion protein), formed by the means of –1 reading frame shift during the process of translation. The replication of the L-A of dsRNA is similar to the replication of the reovirus and complies with a conservative mechanism [18].

Killer viruses (M1, M2, M28, and Mlus) of yeast *S. cerevisiae* have their own capsid. Every virus dem-

onstrates killer activity relative to sensitive strains and killers of the other type; infected strains are resistant to their own toxin [6]. It is interesting that the Mlus virus, detected in the wine strains of *S. cerevisiae*, can inhibit killer activity in the strains of its own type, as well as the yeast of other species: *Kluyveromyces lactis* and *Candida albicans* [19]. In natural strains, the infected cells inherit only one copy of the genome of the killer virus M-dsRNA, as the coexistence of numerous genomes with different virus specificity is excluded at the level of dsRNA replication, probably as a result of competition for the protein of the envelope and the Gag-Pol [6]. The strain with multiple resistance to killers can only be obtained [20] by means of the transformation with multicopy plasmids, which contain complementary DNA coding preprotoxins K1, K2, and K28. Despite the fact that all killer viruses are coded with different dsRNA, are different in size, and have genomes that do not have significant homology, they are characterized with a similar structural arrangement. The size of the killer viruses is half the size of the L-A virus; the virus is packed in capsid, synthesized by the helper virus. The replication cycle of the M virus depends on the L-A virus. Features of replication are studied in detail in the review [21].

The RNA of viruses M1, M2, and M28 only contains the 5'-ORF coding killer toxin and 3'-noncoding area, which play an important role in the replication and formation of the capsid. The sequencing of the 5'- and 3'-areas of their (+) RNA chains demonstrates that homology exists only within a 6-nucleotide sequence at the 5'-terminus, which is probably important for the initiation of the synthesis of the (+) RNA chain. The 5'-terminus of the M1, M2, and M28 (+) RNA chain contains initiation codon AUG in position from the 14^{th} to 16^{th}, from the 7^{th} to 9^{th}, and from the 13^{th} to 15^{th} nucleotide, respectively, which is the beginning of each ORF of the killer toxins [21]. Information about these killer toxins was obtained during the cloning of kDNA and its expression in sensitive non-killer strains. It turned out that, in all three systems, the kDNA expression resulted in the synthesis of the killer toxin and components of immunity; therefore, the killer toxin and the factor that ensures immunity are coded using the same ORF [16].

WEAPONS OF KILLER STRAINS

Killer yeast secretes proteins that are toxic to sensitive organisms within the environment. Toxins differ in their amino acid sequence and affect target cells in different ways. At the same time, the mechanism of synthesis, processing, and secretion are very similar. As a rule, a toxin is coded with one ORF and is synthesized as one polypeptide – a preprotoxin containing potential sites for cleavage with the proteases Kex2p and Kex1p and potential sites for N-glycosylation. Immediately after synthesis, preprotoxin is exposed to post-translational modifications, passing through the endoplasmic reticulum (ER), the Golgi system, and secretory vesicles, which result in the secretion of a mature active toxin [6, 21].

Currently, the ways that killer toxins of many types of yeast secrete and modify are known. Killer toxin K1 (19 kDa) is studied best of all and is secreted in the form of two non-glycosylated subunits: α (9.5 kDa) and β (9.0 kDa), which have a common precursor with a molecular weight of 42 kDa (protoxin). Subunits α and β are connected by a disulfide bond. Toxins K2 and K28 have a similar structure, mainly at the level of their precursor [22] (Fig. 1).

The stages of the killer toxin maturation is studied for K1 and K28 and is discussed in detail in the reviews [6, 21]. The general scheme is presented in Fig. 1.

In the case of the killer toxin K1, the precursor is translated from the RNA of virus M1 (316 a.k.); this is a preprotoxin with a molecular weight of 35 kDa (M1p). The preprotoxin includes an N-terminal leader sequence, which comprises a signal peptide (26 a.k.), followed by subunits of toxin: domain α (102 a.k.) and domain β (82 a.k.) split by central domain γ (86 a.k.), which carries three potential sites of N-glycosylation.

Directed by the leader sequence, or any part of it, preprotoxin enters ER; then, the signal peptide is removed by peptidase, which produces a protoxin by cleaving the peptide bond after ValAla26, in accordance with substrate specificity.

In ER, the γ-domain is N-glycosylated and takes a form that is ready for transportation to the Golgi system for further maturation.

The residual N-terminal segment from 27 to 44 a.k. is probably released into the Golgi system by unidentified proteases of yeast, which cleave the peptide bond after ProArg44.

The endopeptidase, coded by the *KEX2* (Kex2p) gene, is responsible for cleaving the γ-domain of the precursor of the killer toxin after ArgArg149, LysArg188, and LysArg233 residues, which result in the release of the α- and β-subunits of the toxin.

The reaction of this cleaving takes place with different speed or effectiveness in the same vesicles in the late compartments of the Golgi system, probably to minimize the formation of lethal fragments or to increase the production of fragments involved in the immunity development.

Subunit β is further modified with serine carboxypeptidase, coded by the *KEX1* (Kex1p) gene, which removes the arginine at the C-terminus.

Ultimately, the mature toxin K1 is removed from the cell through the secretory pathway of the yeast as a dimer,

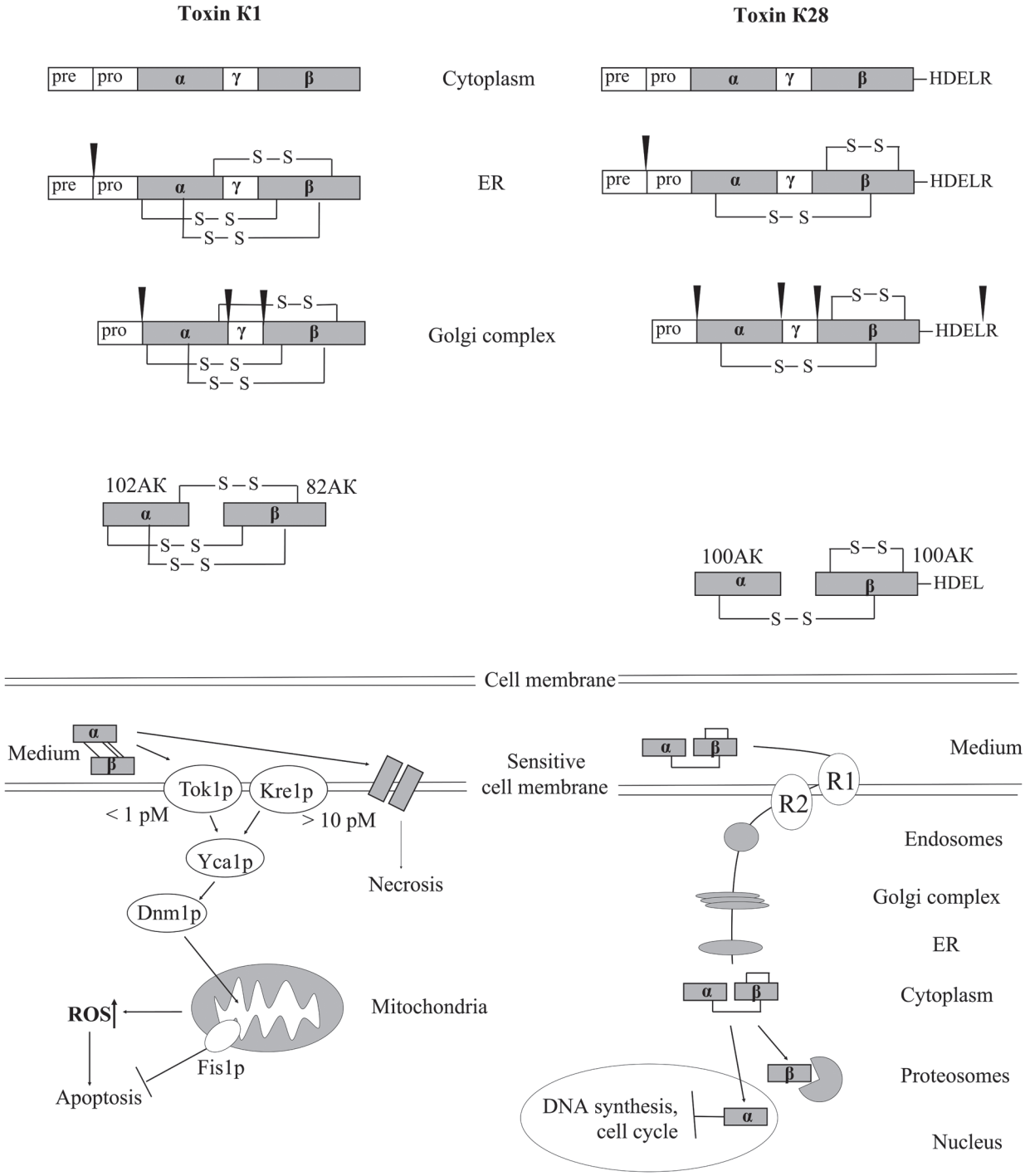

Рис. 1. Stages of maturation and mechanisms of effect of killer toxins K1 and K28. Toxins are synthesized in the form of prepropeptides in cytoplasm, after which they are transported in endoplasmic reticulum (ER). Cleavage of signal sequence(pre-), conversion of disulfide bonds and glycosylation take place in ER. Formed propeptides come to the Golgi system, in which further cleavage of the signal sequence (pro-) and removal of the γ subunit takes place. Mature killer toxins are secreted into the environment. Mechanisms of their effect on sensitive cells are different. Toxin K1 in low concentrations starts the mechanisms of programmed cell death. Acting through proteins Tok1, Kre1, Yca1, and Dnm1, it ultimately leads to an increase of the level of reactive forms of oxygen (ROS) in the cell and activation of apoptosis. At high concentrations, toxin K1 forms channels in the membrane, which results in cell necrosis. Toxin K28 enters the cell by means of endocytosis and by moving backwards along the secretory pathway to the cytoplasm. Its cleavage takes place here into α and β subunits. Subunit α is migrated to the nucleus, where its activity blocks DNA synthesis and cell division

in which both subunits are connected by three disulfide bonds. The mature toxin K28 is also removed from the cell as a dimer, in which both subunits are connected with a disulfide bond between the cysteine residues in the α- (Cys56) and β- (Cys333) subunits. In the β-subunit, there is an intra-chain bond between residues Cys307 and Cys340 [6, 39].

RECEPTORS FOR KILLER TOXINS ON SENSITIVE CELLS AND MECHANISMS OF CELL DEATH

To penetrate the yeast cell, a killer toxin must overcome two barriers: the yeast cell wall and the plasma membrane.

At the first stage, the killer toxin interacts with the primary receptors in the cell wall. Primary receptors for killer toxin K1 are β-1,6-glucans, which are components of the yeast cell wall. It was demonstrated that strains with mutations in genes *KRE1* and *KRE2* were not sensitive to the toxin effect. The *KRE1* gene codes the glycoprotein involved in the assembly of the β-glucans of yeast, and the *KRE2* gene codes the α-1,2-mannosiltransferase involved in the mannosylation of the proteins [23, 24].

The action of the K2 toxin is similar to that of K1; the binding of toxins K2 and K1 with the yeast cell takes place in a similar manner. The K2 toxin saturates the receptors on the surface of the yeast cell for 10 min. The amount of K2 toxin that is bound with the cell of the wild-type yeast can reach 435–460 molecules. It was detected that an increased level of β-1,6-glucan directly correlates with the number of toxin molecules on the cell surface. Poly-β-1,6-glucans such as pustulan, as well as glucans of other types, such as laminarin and chitin, effectively block the killer activity of K2. Thus, primary receptors of the K2 toxin, as well as that of K1, are β-1,6-glucans [25]. The identification of genes involved in the primary binding of killer toxins with the cell wall appeared to be a complicated task, as this interaction depends on many factors, including the synthesis of mannoproteins, the biogenesis of lipids, and the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor of membrane proteins [26].

The primary receptor of the killer toxin K28 is the mannoprotein of the cell wall (185 kDa), a carbohydrate component of which plays the main role in toxin recognition. The removal of the protein part of the mannoprotein does not affect the receptor's ability to bind with the killer toxin K28; the toxin binding was damaged during the hydrolysis of α-1,6-bond of a carbohydrate component. The two mannose residues on the external side of the cell wall are sufficient for exotoxin binding [27]. The binding of the yeast killer toxin K28 with the primary receptor is specifically blocked by polyclonal antimannoprotein antibodies, which mask toxin-binding sites on the surface of sensitive cells. Mutations in the genes of *S. cerevisiae,* disturbing synthesis of mannoproteins such as *mnn1*, affect the structure of carbohydrate chains formed with α-1,3-mannotriose and result in a resistance to the K28 toxin due to the absence of the killer toxin binding sites on the surface of the cell wall [28]. It was demonstrated that subunit β is responsible for the interaction of the killer toxin, K28, as well as that of killer toxins K1 and K2 with the cell wall [29].

It should be noted that primary receptors are specific relative to different killer toxins. Moreover, the chitin of the cell wall of the *S. cerevisiae* yeast can be a receptor for the killer toxin *K. lactis* [30]. Two populations of receptors of the killer toxin K1 which are different in their biochemical properties can even exist on the surface of cells of one strain, [31]. It is supposed that the binding of the toxin with the outside receptor of the cell wall is responsible for its inclusion in the membrane.

The modes of various types of killer toxins (K1, K2, and K28) action on sensitive cells are different, and in accordance with this secondary receptors are also different. As for toxins K1 and K2, at the first stage, the energy-independent bond between toxin molecule and 1,6-β-D-glucan receptor is formed in the cell wall. After this, the killer toxins, K1 and K2, are transferred to a cytoplasmic membrane. Two strictly hydrophobic areas next to the C-terminus of the α-subunit of K1 have alpha-helical structure divided by short hydrophilic segments and can act as the membrane-penetrating domain, which is responsible for the formation of the channel on the cytoplasmic membrane [32]. To identify the secondary receptor of the killer toxin, K1, in the cytoplasmic membrane, the mutants resistant to the toxin at cell level, and the spheroplast level, are obtained for sensitive cells. This is how mutants in the *KRE1* gene were obtained; whose product probably facilitates the fixation of the toxin and promotes the formation of the ion channel [33]. Formation of the energy-dependent complex between the toxin and the Kre1p receptor on the cytoplasmic membrane results in the activation of the membrane channel TOK1, which is identified as a target for the K1 toxin. Ultimately, the membrane's permeability is increased for protons, potassium ions, and high-molecular compounds such as ATP, which cause cell death. While being a toxin receptor, Kre1p does not have any direct relationship with toxicity or immunity. This is confirmed by experiments that studied the properties of the mutants *kre1*Δ transformed with plasmid containing α-subunit of K1. The synthesis of α-subunit of K1 in the sensitive strain of yeast fully simulated the cell treatment with an exogenous toxin. The simultaneous production of protoxin resulted in the development of immunity in transformants to α-subunit of K1, despite the fact that α-subunit cannot be transported to ER and, therefore, is unable to interact with the toxin precursor. The obtained results suggest that

the development of immunity to K1 can involve an unidentified protein-effector [34].

In general, the K2 toxin has the same effect as K1. The deletion of the *KRE1* gene disturbs the interaction with the endoplasmic membrane of the K1 toxin, as well as K2; this indicates that the Kre1 protein is a receptor for both toxins. However, mutations in the other genes (*GDA1*, *SAC1*, *LUV1*, *KRE23*, *SAC2*, *KRE21*, and *ERG4*) affected the sensitivity of the cells to K1 and K2 in a different way. Factors, whose changes result in resistance and hypersensitivity to toxins, are also different. Seventy percent of effectors of K2 differ from the effectors of K1 and K28; besides, under the effect of K2, ATP does not move outside the cell. Thus, every killer virus develops its own way of affecting sensitive cells in the process of evolution [35, 36].

The effect of the killer toxin, K28, differs from the effect of K1 and K2. Details of toxin penetration into a sensitive cell, the way of signal transfer inside the cell, and the connection of the toxin's effect on signal cell pathways were studied in the work [37]. At the first stage, the K28 toxin interacted with the receptors of the cell wall – mannoproteins – which is probably is a prerequisite for further toxin interaction with cytoplasmic membrane. In the work of Schmitt et al., the HDEL sequence was identified at the C-terminus of the β-subunit of the mature toxin [38]. The secondary receptor for K28 is the Erd2p, which recognizes the signal sequence H/KDEL. This is supported by the following facts: both spheroplasts and cells *erd2*Δ are resistant to the toxin; Erd2p is localized in membranes of the early secretory pathway, as well as in the cytoplasmic membrane, in which it binds H/KDELproteins such as the K28 toxin, GFP-KDEL, and Kar2p. It is interesting that the KDEL human receptors fully recover sensitivity to the toxin in the absence of endogenous Erd2p [39].

The mutant K28, lost sequence HDEL, at the C-terminus of β-subunit becomes nontoxic and loses its ability to penetrate the cell. Complex K28/Erd2p, by means of endocytosis, enters the early endosomes; then using the secretory pathway and retrograde transport, it goes into the cytoplasm. It was demonstrated that the binding of HDEL in relation to toxin K28 and Erd2p depends on the pH. At pH 4.7, K28 is bound with the receptor Erd2p placed in the endoplasmic membrane. Complex in the bound state goes through endosomes and the Golgi system (pH 6.0–6.2) and reaches ER (pH 7.2). The regulation of this process is unknown, and the process of the prevention of complex degradation in vacuoles is unclear. The vacuolar defects contribute to the toxin transportation to ER [40]. The number of proteins that are required for anterograde and retrograde transport of cell proteins and the K28 toxin was identified [41].

As already mentioned, the killer toxin, K28, is secreted as α/β-heterodimer that kills sensitive yeast cells using a receptor-mediated way and blocks the DNA synthesis in the nucleus [42]. Yeast mutants *end3* and *end4*, the cell lost receptor HDEL, and mutants with defects of protein transport from the Golgi system to ER (*erd1*) are resistant to the toxin; because of the background of these defects, the toxin cannot enter the cell and move by the secret pathway in the reverse direction. Site-directed mutagenesis confirms that the HDEL motif of the β-subunit of the toxin ensures the retrograde pathway. It is interesting that the HDEL motif is initially hidden with the arginine residue (HDELR) in the toxin-secreting yeast; the cleavage of this amino acid is done with Kex1p in the later cisternaes of the Golgi system. The inhibition of the work of Kex1p specifies a high level of secretion of biologically inactive protein, which cannot repeatedly enter the secretion pathway. The export of the toxin from ER to cytoplasm is mediated by translocon Sec61p and requires the availability of the functional ER chaperones Kar2p and Cne1p [39]. After entering the cytoplasm, the complex α/β dissociated and the β-subunit is ubiquitinated and sent for degradation in the proteasome. Subunit α enters the nucleus and kills the cell causing the irreversible termination of cell cycle at the G1/S stage, inhibiting the DNA synthesis [42].

The fate of the sensitive cell after the interaction with the toxin is determined by the features of its action. Killer K1 toxin in the sensitive cells of yeast disturbs the function of the cytoplasmic membrane through the formation of lethal ion channels; K28 causes cell death by blocking the replication of DNA [42]. Regarding the mode of action for K28, two dose-dependent mechanisms were identified. The low concentration of the toxin (<1 pM) causes apoptosis with typical markers: DNA fragmentation, chromatin condensation, and the appearance of phosphatidylcholine on the outer side of the cytoplasmic membrane. This toxin action depends on the yeast caspase (Yca1p) and is accompanied by the appearance of reactive forms of oxygen. Toxin concentration can be increased in laboratory conditions (>10 pM), which results in the necrosis of cells and the blocking of the cell cycle at the G1/S stage; the cells have a middle-size bud with one nucleus in the mother cell and 1n of DNA. As it was already mentioned, the K28 toxin irreversibly disturbs the DNA synthesis [42–44]. In natural conditions, cells deal with the low concentration of the toxin, and apoptosis is the main mechanism of cell death.

THE NATURE OF THE IMMUNITY OF KILLER STRAIN CELLS

The genome of the M virus ensures the synthesis of the toxin that determines the toxicity in relation to sensitive cells and immunity in relation to its own to-

xin [45]. It is interesting that receptors for toxins are existed on the surface of the killer strain cell. Nevertheless, the killer cell has an immunity to its own toxin. The up-to-date model of immunity is developed for K28. The killer toxin, K28, penetrates its own cell, and, by means of the gradient of the pH in the cell (from 4.5 to 7.2), α/β thiol groups in the heterodimer are re-arranged. This results in the formation of inactive toxin trimers, tetramers, and oligomers, which is proven in *in vitro* experiments. The formation of these oligomers *in vivo* is prevented by ER chaperones and protein disulfide isomerase Pdi1p, which retain a mature toxin in the heterodimeric conformation [40]. Pdi1p of yeast, similar to the human enzyme, has disulphidisomerase and oxidoreductase activities and contains two catalytic thioredoxin domains; the sequence Cys–XX–Cys and the domain responsible for protein–protein interactions are located in the active center. Deletion mutants of the *pdi1* yeast are unviable. The absence of Pdi1p can be partially compensated of the Eug1p and Mpd1p overproduction[46].

The process of the formation of disulfide bonds includes the number of sequential thiol–disulfide exchange reactions, in the course of which the temporary complexes of protein disulfide isomerases with processed proteins are formed. At least two protein disulfide isomerases, Ero1p and Pdi1p, are involved in closing of the S-S bonds of secretory proteins of yeasts. At the first stage, Ero1p interacts with Pdi1p and oxidizes it; then, the oxidized form of Pdi1p binds to the protein-substrate, and oxidizing it initiates the formation of disulfide bonds [47]. The correct closing of disulfide bonds stabilizes the native structure and has a decisive value for the secretion of some proteins. It is supposed that Pdi1p induces some additional unknown structural changes in K28, which can promote toxin transfer to the cytoplasm. In the absence of Pdi1p, the α/β-heterodimer oligomerizes, which may promote the advanced release of cytotoxic α-subunit. Killer strains have a quick degradation mechanism of own killer toxin when enters the host cell. After the retro-translocation in the cytoplasm, the α/β-heterodimer of the toxin forms a complex with unprocessed precursor, which is further exposed to polyubiquitination and sent for degradation [39].

GENETIC CONTROL OF YEAST SENSITIVITY TO KILLER TOXINS AND THE HOST CELL'S RESPONSE TO KILLER VIRUSES

An original work was devoted to the analysis of the effect of the killer toxin, K1, on the gene expression of genome of yeast *S. cerevisiae* [48]. To study the sensitivity towards the killer toxin, K1, which is bonded with the β-glucans of the cell wall, and formes pores in the cytoplasmic membrane, the deleted mutants of 5718 genes were used. Sensitive and resistant mutants were detected with a more-or-less expressed effect. Pleiotropic effects were detected in some mutants that were resistant to toxins: sensitivity to detergents, hygromycin and Calcofluor-White, fluorescent dye bonded with the chitin of the cell wall. Genes, for which mutations affected the sensitivity towards the killer toxin, K1, controlled the synthesis of the glucans and mannoproteins of the cell walls, secretory pathways, biosynthesis of lipids and sterols, and signal transduction [48].

An interesting approach was used by Santos et al. to study a transcriptional response of cells of yeast *S. cerevisiae* to killer toxin PMKT *Pichia membranifaciens*. It turned out that the yeast cells' response to toxin action is very similar to the response of the cell, which adapts to ionic or osmotic stress. The signal was transmitted through the high osmolarity glycerol (HOG) pathway, and the phosphorylation of Hog1p took place in response to the toxin action, which indicated the existence of the common mechanisms of cell response to killer toxins and the regulation of the ion homeostasis [49].

It is known that the yeast killer toxin, K28, and ricin and the cholera toxin are attributed to the family of A/B toxins, which penetrate cells by means of endocytosis with a further retrograde toxin transfer to the cytoplasm. For this reason, the study of the genetic control of the yeast cell response to the K28 toxin is of special interest for medicine. In 2000, the search for mutants resistant to the K28 toxin resulted in the detection of mutants *sla2* and *erd2* [37]. Protein Erd2, as previously mentioned, is an integral protein of the membrane and directs toxin transportation from the Golgi system to the ER of the target cells, whereas Sla2p is an adaptor protein, which binds the actin, clathrin, and the endocytosis process. Sla2p is involved in the assembly of cytoskeleton, the polarization of cells, and is presented in actin cortical patches in the emerging bud. In the later work by Carroll et al., more than 5,000 deletion and ts-mutations of resistance and hyper-sensitivity to the K28 toxin were analyzed. Mutations that result in the resistance to K28 occurred in genes controlling the biogenesis of the cell wall (33 genes), of the plasmic membrane (18), the endocytosis (21), the transport (16), the mitochondria (14), and the cell cycle (13). Mutations that resulted in hypersensitivity occurred, as a rule, in the genes involved in the formation of ribosomes and translation (53 genes), RNA processing and RNA transport, transcription, and ionic homeostasis. It was detected that the deletions in the genes that coded all four subunits of the clathrin's AP2 adaptor (*apl1*Δ, *aps2*Δ, *apl3*Δ, and *apm4*Δ) resulted in resistance to K28. Thus, sensitive cells can, or at least try to, resist the killer toxin action [50].

To obtain a response to the question of how viruses in the cell affect the host gene's expression, the changes in the transcriptome of strains with the L-A and M1 viruses were compared with strains without viruses. Special attention was paid to the genes, which mutations affected such processes as the antivirus system required for the degradation of dsRNA viruses, the control of the replication of dsRNA, protein processing, secretion, the synthesis of proteins of the cell wall and signaling, and the inhibition of apoptosis [51]. It turned out that the presence or absence of the L-A and M viruses in the cell insignificantly affected the expression of the genes of the host cell. Similar results were obtained in the study of the transcriptome of the strain of the *S. cerevisiae* lost virus M2 and retained virus L-A and of the strain that lost both viruses. Overall, 486 genes were identified; the expression of which changed after the loss of the M2 virus; the expression for 715 genes changed as a result of loss both viruses. The expression of the major part of the genes was increased or reduced a minimum of one and a half time but, as a rule, not more than four times. A transcription of only 12% of the differentially expressed genes of the strain without the M2 virus, as well as the strain that lost both viruses, was changed four times or more. These genes controlled the biogenesis of ribosomes, the functions of the mitochondria, the stress-response, and the biosynthesis of lipids and amino acids, which probably indicated a change in energy consumption for the protein and RNA synthesis in strains without viruses [52].

EVOLUTION OF KILLER SYSTEMS

The L-A virus is required to support the satellite virus M, which contains information about the toxin in its genome and ensures the immunity of the host cell. From the evolutionary point of view, such relationships can be a system of "auto selection," i. e., the selection of microorganisms in terms of adaptation properties. Killer cells obtain advantages due to virus presence; however, these advantages are relative. The death of adjacent cells requires the high concentration of the killer cells compared with the concentration of sensitive cells. If toxin-coding elements provide selective advantages to the host cell, such relationships shall be fixed during evolution and widespread in the population. However, killer strains in natural populations are few; therefore, toxin-coding elements are not very effective. It is likely that only the absence of the RNA-interference system allows them to be kept; viruses are selected in hosts that, for any reason, have lost RNA interference [53]. It turns out that the killers' phenotypes are kept only in cells in which *SKI* genes are disturbed. Proteins Ski2p, Ski3p, and Ski8p block expression of non-polyadenylated mRNA, such as virus dsRNA [54].

It is known that evolutionary-conservative 5′3′-exonuclease Xrn1p (Ski1p) together with exosome is involved in the eukaryotes' degradation of cellular mRNA. As virus mRNAs do not have canonical sequences at the end of molecules, such as cap and poly-A-tail, they can be exposed to degradation by these exonucleases. Yeasts use this mechanism as a tool for protection against viruses. Rowley, along with co-authors, detected a high accurate mechanism of specific interactions between virus L-A and exonuclease Xrn1p in different types of yeast. They demonstrated that under the effect of natural selection, the sequence of the *XRN1* gene in the yeast of the *Saccharomyces* species diverged and significantly differs across various species. Changes of the sequence of gene *XRN1* specify different interactions of exonuclease with the L-A virus. Every variation of Xrn1p is adapted to a certain virus. The authors suppose that Xrn1p coevolved with totiviruses, increasing its antivirus activity and limiting the reproduction of viruses in yeasts. Further research demonstrated that Xrn1p interacts with the protein Gag of viruses' envelopes. This fact certifies more complicated interaction between the yeast cell and virus rather than the simple nuclease cleavage of the RNA virus [55, 56]. Thus, the absence of the RNA-interference system in yeasts is probably compensated during the process of evolution with other mechanisms. A probable mechanism of eliminating viruses in yeasts was proposed in the work by Suzuki et al.; they discovered the prion-like element [KilD] in the cell, which contained the M virus and resulted in the hypermutability of virus genome and its inactivation. The nature of this phenomenon has not yet been studied [64].

At the same time, the role of killer systems in the formation of species diversity under conditions of geographic isolation (sympatric speciation), within one ecological niche, is minimal [21]. The coevolution of the L-A and M viruses is of special interest. When nucleotide sequences of the dsRNA of L-A were studied, at least four natural variants were detected. Analysis demonstrated that they were 24% different from each other [58]. The authors demonstrated that the selection of effective pairs of helper viruses and killer viruses resulted from coevolution, and the viruses' pairs are specifically adapted to each other. Thus, in the wine killer strain, K2, only the L-A-2 virus was required for the reproduction of the M2 virus; no other form of L-A virus or L-A-2 ensured the development of the M2 virus at the same genetic background. The genome of other totiviruses, L-BC, which are frequently observed with L-A, is less variable (only 10% of the nucleotides are different). Thus, the "killer" phenotype depends on two types of dsRNA viruses, while each helper L-A virus, coding proteins of capsid for the M virus is specific for its own killer virus, which proves their coevolution. Many authors consider the interaction of yeast cells and mycoviruses as

a form of symbiotic relationship. However, we, as other authors [59], believe that the use of such terminology in this case is disputable because, so far, there is no uniform opinion about whether the viruses are living creatures or not. Besides, the number of issues need to be resolved. What is the method of evolution for the L-A virus and the M1, M2, and M28 viruses, which are almost parasitized on the helper virus? Why are viruses that produce toxins not completely eliminated from the population if they are easily lost and can only be recovered as a result of crossbreeding, which is very seldom for yeasts in natural conditions? Is the availability of the virus in the cell an advantage? Is it connected with the absence of the RNA interference in the yeasts?

THE SYSTEM OF RNA INTERFERENCE AND RNA-CONTAINING VIRUSES

The of RNA interference system is one of the most important systems involved in the regulation of gene expression, as well as cell protection against foreign nucleic acids. RNA interference is based on the suppression of gene expression at the stage of transcription or translation with the active involvement of small RNA molecules. These RNAs generally belong to two main classes: microRNAs (miRNAs) and small interfering RNAs (siRNA).

The molecular mechanisms of RNA interferences modulated by miRNAs were studied in detail in nematodes *Caenorhabditis elegans* [60]. It was demonstrated that miRNAs can be coded in the genome of the organism in the form of individual genes, as well as within the introns of other genes. The synthesis of miRNAs from appropriate genes is usually carried out by RNA-polymerase II. The other mechanisms of miRNAs-production are by means of RNA-polymerase III or from RNA fragments cut during intron splicing [61]. The main pathway of miRNAs biogenesis comprises several stages and starts with the synthesis of the primary transcript (primiRNA), which contains one or several double-stranded hairpins. At the next stage, the ends of transcript are shortened by means of the complex, of which the main components are RNAase III (also known as Drosha) and RNA-binding protein (DGCR8 of human, Pasha in *C. elegans*, and *D. melanogaster*) [62]. The shortened hairpins (premiPHK) are exported through the nuclear membrane to cytoplasm. Other RNAase III (Dicer) interacts with them here, and cuts short double-stranded fragments from hairpins. After the strands are split by helicase, the formed miRNA is included in the complex called RISC (RNA-Induced Silencing Complex). The main components of such complexes are proteins of the *Argonaute* family, which demonstrate endonuclease activity. The specific binding of the RISC complex with the mRNA-target, which is ensured by miRNA, results in the termination of translation processes on this mRNA but seldomly relates to its cleavage [63].

In contrast to miRNA, the biogenesis of small interfering RNAs is connected to the enter of foreign nucleic acids into the cell enter to RNA-containing viruses. dsRNA, synthesized in the process of viruses replication, is cut into short fragments by one of the components of the RNA-interfering system: the endonuclease Dicer. Small interfering RNAs, formed in the process of the cleavage of these fragments, interact with the RISC. In this case, virus RNAs are effectively cleaved by RISC, preventing replication of the virus in the cell. The participation of small interfering RNAs in the regulation of genes at a transcription level by remodeling the chromatin is demonstrated (in particular, in *Schizosaccharomyces pombe*). The original dsRNA is synthesized on an mRNA basis through the RNA-dependent RNA-polymerase (RdRP) coded in genome of *S. pombe* [63].

The system of RNA interference probably arose among the early ancestors of eukaryotes and is rather conservative in fungi. However, the comparative analysis of fungi genomes demonstrates that all budding yeasts lost one or several genes encoding components of the system [64]. The yeasts *S. cerevisiae*, *Candida glabrata*, *K. lactis*, and *Ashbya gossypii*, among the analyzed species of budding yeasts, lost all gene-coding homologies to Argonaute, Dicer, and RdRP. At the same time, representatives of the other yeast species such as *Saccharomyces castellii*, *Kluyveromyces polysporus*, *C. albicans*, and *Candida tropicalis* have the genes encoding protein Argonaute, whereas there are no gene-coding Dicer homologues in these species. The introducing of the genes encoding the proteins of the RNA-interference system into genome of *S. cerevisiae* results in its partial recovery [64]. The presence of RNA interference in yeast *S. cerevisiae* probably allows the existence and functioning of killer viruses in their cells, which can ensure the competitive advantage of the host cell.

The human system of RNA interference is involved in the regulation of 30% of genes at a minimum [65]; therefore, the possibility of losing such a system in living organisms in the process of evolution, as in the ancestors of budding yeasts, is of great interest. The absence of RNA interference in the *S. cerevisiae* yeast makes it a unique model for the examination of molecular pathways of virus transfer and their action in eukaryotes cells [66].

ROLE OF KILLER STRAINS IN NATURAL POPULATIONS

Toxic interactions are probably common and ecologically important for microbes. The excretion of antimicrobial compounds acting against related or associated species is known for bacteria and yeast. So far, the killer strains have been detected among representatives of different

yeast species within the living environment. Eleven species of killer toxins that were identified, are produced by representatives of species such as *Hanseniaspora*, *Pichia*, *Saccharomyces*, *Torulaspora*, *Ustilago*, and *Williopsis*, which take various ecological niches [21].

Marine yeast, *Metchnikowia bicuspidata* WCY, acts as a pathogen in crab mariculture, *Portunus trituberculatus*, but can be eradicated by killer toxins. Screening of sea water, sediments and silt, intestines of sea fish and marine algae, revealed 17 yeast strains, which secreted a toxin into the environment and were capable of killing pathogenic yeast. The strains with highest killer activity were classified as *Williopsis saturnus* WC91-2, *Pichia guilliermondii* GZ1, *Pichia anomala* YF07b, *Debaryomyces hansenii* hcx-1, and *Aureobasidium pullulans* HN2.3. Killer toxins of these yeasts acted against the yeast *M. bicuspidata* WCY, which is pathogenic for crabs. It was demonstrated that the best temperatures for the killer toxin production coincide with natural conditions of the crabs' living environment. The best pH range varied from 4.5 to 6.0. However, the maximum level of toxin synthesis in the analyzed killer strains was observed at different NaCl concentrations. Thus, the change in salt balance, pH, and temperature probably provides advantages to killer strains in their struggle to find an ecological niche [67].

Killer strains comprise a significant part of yeast communities living in rotting plant stems and fruits. Conditions including low pH and high sugar content promote this. When microbial communities are formed, especially at the early stages, the weapon of the killer yeast provides a selective advantage as it prevents the development of competitive microorganisms. The concentration of the toxin produced by strains in natural conditions is low; therefore, it can be supposed that the death of sensitive cells under effect of the toxin takes place mainly through apoptosis [68].

It is known that virus transfer in yeasts can go vertically, in the case of budding, as well as horizontally, in the case of crossbreeding or cytoduction. Yeast viruses are never emitted into the environment. Infected cells are transferred by different species of animals within the area. The safekeeping of killer yeasts in natural conditions is promoted by reduced ambient temperatures in relation to the best temperature for living yeast. It is interesting that many isolates of killer yeasts extracted from fruits quickly lose their killer phenotype, probably due to their cultivation in laboratory conditions at higher temperatures [69]. In natural communities, killer strains and strains sensitive to them coexist, which evidences the spatial splitting of local habitats and the temporary splitting of the development stages, despite the use of the common substrate [70]. The elimination of the competitor usually means its death or the reduction of its viability through the locking of the cell cycle or damage to the membrane's integrity. The availability of the toxin can be an advantage in the struggle between populations with similar ecological demands; the killer strain is then predominant in the community and fully displaces the sensitive strain. However, in nature, these strains successfully coexist and this requires explanation. Toxin production is an example of interference competition. This is a type of ecological relationship, when one or different species do not directly interact; the active suppression of the competing population takes place rather than the simple depletion of common limiting resources. Active interference from a biochemical point of view allows the toxin-producing population to use a significant amount of common resources. Toxin production can be decisive in the fight between populations that require similar living conditions. In certain conditions, killer yeasts can become predominant. At the same time, different outcomes of such relationships are observed in nature: sometimes killer strains are predominant, and sometimes sensitive strains displace killers [71]. It can be supposed that each type of strain has its own niche that is limited to appropriate living conditions. This case can be explained by the rule of competitive exclusion that was formulated by G.F. Gauze for ecologically close species; however, it can be used for this particular case. Experiments demonstrate that when competition for the main resources cannot be avoided, weaker competitors are removed from the community. In the case of the coexistence of sensitive and killer strains, this does not happen probably due to the adaptation of sensitive strains to higher temperatures and environment acidity. It should be considered that some killer strains lose the RNA virus at a high temperature and become either a non-killer or a neutral yeast.

PRACTICAL USE OF KILLER TOXINS

Killer toxins are widely used in the food industry, in agriculture and aquaculture as a bioprotective agent, and in medicine. The yeast *S. cerevisiae* is traditionally used in the food industry. For the first time, killer toxins (mycocins) were detected in strains of the yeast species that were used for brewing. Killer yeasts frequently occur among those strains that exist in conditions of high cell density and tough competition for substrates, for example, during grape juice fermentation and olive pickling. Under these conditions, the killer toxin is aimed at the wide range of unrelated microorganisms. Killer yeast is used as the starter culture in wine production; the yeast propagates itself and suppresses the growth of pathogenic and other harmful microorganisms. Some yeasts, such as *Kluyveromyces wickerhamii*, produce killer toxins against *Dekkera* and *Brettanomyces* yeasts, which cause the unpleasant

smell during wine fermentation. In the milk industry, killer yeast is used as the starter culture to prevent damage to cheese, yogurt, and other dairy products. This review presents the detailed use of killer yeast within the food industry [72]. Killer yeast can be also useful within aquaculture. Killer toxins of marine yeast can be used to effectively fight crab disease have a negative effect on the pathogenic yeast, as well as on the crabs [67].

Killer factors are used for plant protection. A significant problem here are the diseases related to citrus plants, which are caused by the fungus pathogen *Geotrichum citriiaurantii*. As the chemical compounds that are certified for fighting this pathogen are absent, harvest loss can be considerable. Killer toxins can become an alternative method of fighting against this pathogen. Among the yeast isolated from the surface of leaves and citrus fruits, growth of pathogen was most effectively suppressed by strains of *Rhodotorula minuta, Sporobolomyces koalae, Candida azyma, S. cerevisiae, Rhodotorula mucilaginou*s and *Aureobasidium pullulans*, which synthesized killer toxins. The active mechanism of these killer toxins has not been studied yet; however, preliminary data state that its target is the component of the cell wall of *G. citriiaurantii* [73].

Toxins produced by yeast can be used in medicine. When the killer activity of natural variants of wild yeast was studied, it was determined that 5–30% of strains can kill the standard sensitive strain of *Candida glabrata* [70]. Deep knowledge of mechanisms of the activity of killer toxins allows the creation of a new generation of antimicrobial agents, which will fight microbial infections resistant to antibiotics and other standard medicines. Medicines based on killer toxins can be useful for the treatment mycoses of humans and animals. The principle of the action of such medicines consists in the hydrolysis of the β-1,3-glucan of the cell wall of pathogenic yeast. However, the direct use of killer toxins can be limited. As they are glycoproteins, they can be antigens and cause an immune response. The pH for the best activity of the killer toxin from different sources does not always coincide with the pH within the body; therefore, the search for natural killer toxins with a wide range of activity, working with a different pH, continues.

A new prospective area of use for killer toxins is the obtainment of an anti-idiotype antibody. Anti-idiotype antibodies reproduce a configuration of antigenes and can be considered as their analogues. Thus, biological activity of fragments of killer toxin PaKT was studied, which demonstrated antimicrobial activity against *C. albicans*, *Pneumocystis carinii,* etc. It was not possible to use a killer toxin directly for treatment as it demonstrated activity within a narrow range of pH and temperature, which differed from the physiological values and was toxic and immunogenic. Monoclonal antibodies were obtained, which could neutralize killer toxin activity. This was the basis for the production of anti-idiotype antibodies that could compete with killer toxins for binding sites and activity related to *C. albicans*. These so-called antibiobodies (antibioticlike antibodies) had a direct antifungal effect without any additional factors; they operated at pH and temperature physiological values and had no immunogenicity or toxicity. Antibiobodies are prospective class of compounds for the treatment of different human diseases [74].

CONCLUSIONS

Antagonism is widespread among microorganisms and is specified by the production of different antimicrobial substances. The ability to synthesize and secrete toxin proteins was detected in many yeast species. The structure and properties of toxins and the mechanisms of their actions can differ within species. The study of the phenomenon of killer activity in the model organism, yeast *S. cerevisiae*, resulted in significant progress in many areas of biology. It allowed to obtain important information about the mechanisms of the interaction of the virus and cells of yeast, the system of virus support in individual cells, and in the population, and revealed the issues of the evolution and co-evolution of biological systems. As the killer toxins resemble natural secreted proteins or glycoproteins, the detailed analysis of their structure and synthesis have significantly expanded our understanding of the mechanisms of the post-translational modification of eukaryotic proteins during the secretion process. The analysis of the receptor-mediated mode of killer toxin action appeared to be an effective tool for the study of molecular structures and the assembly *in vivo* of the cell walls of yeast. The prospective area of use of the yeast killer toxins is the production of new medicines for the treatment of fungal diseases caused by pathogenic strains of yeast *C. albicans*. Besides, killer strains and yeast killer-toxins are used within the food industry as an effective tool to fight pathogenic microorganisms in the process of wine production, brewing, and baking. Considering that a major part of the currently known killer yeasts are not examined in detail; it is likely that new properties and areas of use for yeast killer toxins will be determined.

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