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REVIEW ARTICLE

D. Marquina · A. Santos · J.M. Peinado

Biology of killer yeasts

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Abstract Killer yeasts secrete proteinaceous killer toxins lethal to susceptible yeast strains. These toxins have no activity against microorganisms other than yeasts, and the killer strains are insensitive to their own toxins. Killer toxins differ between species or strains, showing diverse characteristics in terms of structural genes, molecular size, mature structure and immunity. The mechanisms of recognizing and killing sensitive cells differ for each toxin. Killer yeasts and their toxins have many potential applications in environmental, medical and industrial biotechnology. They are also suitable to study the mechanisms of protein processing and secretion, and toxin interaction with sensitive cells. This review focuses on the biological diversity of the killer toxins described up to now and their potential biotechnological applications.

Keywords Killer yeasts · Mycotoxins · Genetic basis · Killer toxin · Virus-like particles

Introduction

Many yeast cultures secrete killer toxins that inhibit growth of other yeasts strains, but to which they are immune. Killer (K), sensitive (S), and neutral (N) phenotypes of *Saccharomyces cerevisiae* were first described by Makower and Bevan [16]. Killer yeasts were thereafter shown to be widespread amongst laboratory strains. These early observations led to the search for killer yeasts in other genera.

The assay method currently employed to distinguish killer strains was first described by Makower and Bevan in 1963 [16]. Woods and Bevan refined this method and established the optimal culture conditions for toxin production and activity [44]. The killing ability of these compounds may be underestimated or may even remain unnoticed depending on the selection of the appropriate sensitive strain and other experimental conditions. For example, the addition of sodium chloride, which enhances the killer phenotype of some strains, showed that some supposedly resistant strains were sensitive to toxin [15]. The best studied killer species is *S. cerevisiae*, which secretes different types of killer toxins.

D. Marquina (✉) · A. Santos · J.M. Peinado
Department of Microbiology III,
Biology Faculty, Complutense University of Madrid,
28040 Madrid, Spain
E-mail: dommarq@bio.ucm.es
Tel.: +34-913944962
Fax: +34-913944964

Saccharomyces cerevisiae killer system

When the first studies on the nature of the killing phenomenon showed the involvement of cytoplasmic non-Mendelian genetic determinants, the occurrence in killer yeasts of double-stranded RNA (dsRNA) associated with virus-like particles (VLPs) was suggested [1, 8, 19, 23, 29,37]. *Saccharomyces cerevisiae* killer toxins (K1, K2 and K28) are encoded by different satellite dsRNAs (M1, M2 and M28) that are cytoplasmically inherited and encapsidated in virus-like particles. For their replication and encapsidation, another group of helper yeast viruses (L-A) is needed [30, 34, 42,43]. These well-characterised dsRNA viruses are members of the family *Totiviridae*. The presence of a satellite M dsRNA in cells coinfected with an L-A virus is responsible for the killer-immune phenotype observed in the killer strains [2,45].

A single open reading frame encodes the toxin, which is synthesized as a single polypeptide pre-protoxin comprising hydrophobic amino termini larger than those usually found on secreted proteins and potential kex1/kex2 cleavage and N-linked glycosilation sites. The pre-protoxin, once synthesized, undergoes post-translational modifications via the endoplasmic reticulum, Golgi apparatus, and secretory vesicles, resulting in the secretion of the mature, active toxin (Fig. 1). The N-terminal leader directs the precursor protein to the endoplasmic reticulum functioning as a conventional

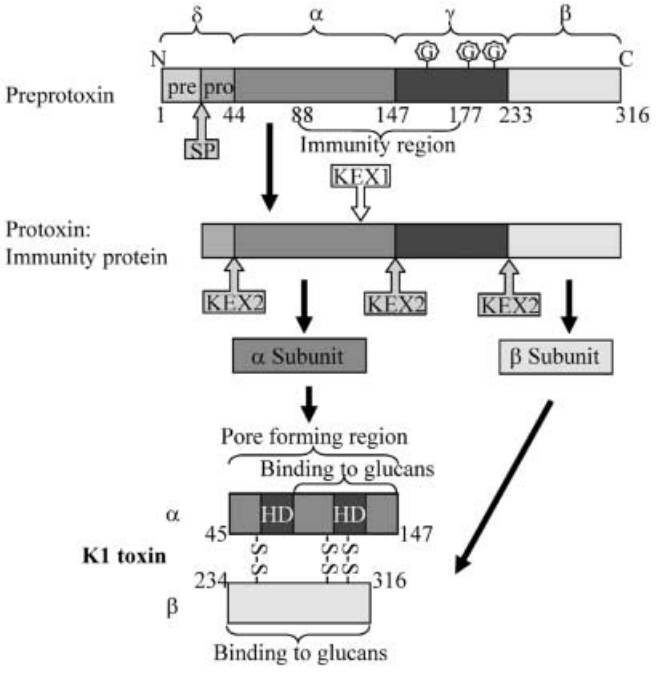

Fig. 1. Outline of the structure of the K1 killer toxin-immunity precursor and structure of the mature toxin. *SP* Signal peptidase, *KEX* killer expression proteases involved in toxin processing, *G* location of the glycosylated asparagine residues, *HD* hydrophobic domains [42, 43]

signal sequence. This molecule is a protoxin that has been shown to be converted into a 34 kDa species by the action of endoglycosidase H; therefore, it is a glycoprotein. Glycosylation is necessary for the efficient secretion of toxin [3]. The active K1 killer toxin, however, is not glycosylated; thus the glycosylated portion must be removed during the toxin maturation process. The 34 kDa protoxin contains all peptide components of the K1 killer toxin. When the protoxin passes through the Golgi apparatus, a proteolytic cleavage occurs, and both α and the β disulfide-linked toxins form and are released by fusion with the plasma membrane. When the toxin has been secreted from the plasma membrane, two processes reduce the amount of toxin present in the extracellular medium: (a) the activity of (phenylmethylsulfonylfluoride-sensitive proteases from the cell, which degrade a substantial proportion of the secreted toxin molecules [42], and (b) the presence of receptors for the killer toxin on the cell wall of the killer strain. (1→6)-β-D-Glucan in the cell wall plays a major role in the toxin's binding to sensitive cells [9, 27,28]. Killer cells are immune, but have these linkages. Some secreted toxin is bound and is not found in the medium.

As described above, the K1 killer toxin secreted by *S. cerevisiae* consists of a disulfide-linked α-β dimer. Both subunits have a relatively high content of charged and hydrophobic amino acids. The excess charge of toxin (pI = 4.5) resides in the β subunit. The toxin is capable of forming multimers of the basic dimer. Some studies have described the presence of multimers up to octamers; it is not clear, however, whether these multimers are necessary for toxin action. Based on the dimeric structure of the killer toxin, several modes of action are possible. The most widely accepted model suggests that the α-subunit, with two hydrophobic, potentially membrane-spanning domains, is a candidate for the channel domain. This subunit might provide the proton-binding sites for the cation channels produced by toxin in target cell membranes. In this model, the β-subunit is involved in cell-wall receptor binding [18] (Fig. 2).

Other killer toxins

The major killer strains of *S. cerevisiae* belong to the killer classes K1 and K2, which kill each other, but are immune to killer toxins of their own class. The activity of K1 killer remains stable within a narrow range of acidic pH, is unstable at temperatures above 25 °C, and can be inactivated by agitation. Soon after the discovery of this phenomenon it became obvious that killer strains, in addition to not being restricted to *Saccharomyces*, could also be found in other genera

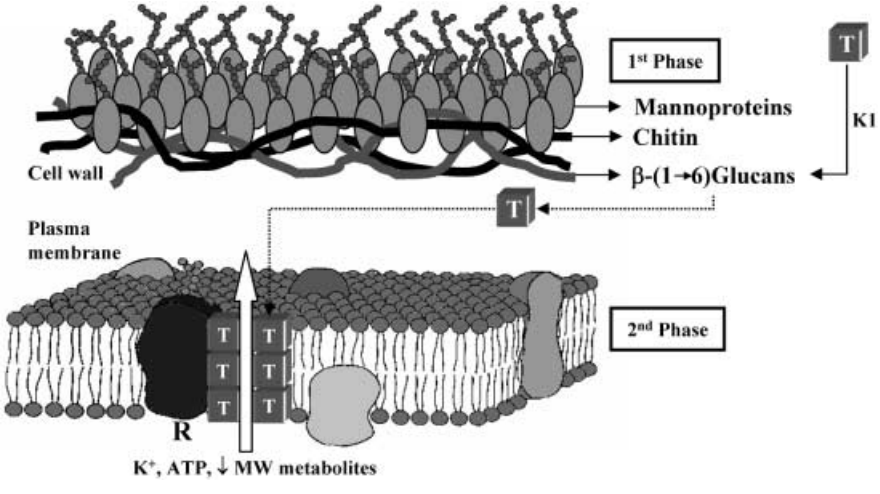

Fig. 2. Receptor-mediated killing of a sensitive yeast cell by K1 killer toxin of *Saccharomyces cerevisiae*. After binding to the cell wall [9], K1 killer toxin is transferred to the cytoplasmic membrane and acts by forming voltage-independent cationic transmembrane channels, which cause ion leakage and subsequent cell death. The existence of a receptor (*R*) in the membrane has been postulated

including *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Sporidiobolus*, *Tilletiopsis* and *Zygosaccharomyces* (Table 1). Subsequent studies of a variety of killer toxins showed that the characteristics of the K1 killer toxin and other toxins tend to be similar. Toxins from all killer strains studied are protease-sensitive, heat-labile macromolecules. Most of them are stable and act only at acidic pH values. This is the case for *Cryptococcus humicola* (killer toxin active at pH 3–5.5) [6], *Pichia kluyveri* (killer toxin active at pH 2.5–4.7) [27] and *Pichia inositovora* (killer toxin active at pH 3.4–4.2). The most stable killer toxins are those of *Hansenula mrakii* (stable at pH 2–11, and unaffected by heating at 60 °C for 1 h) [36] and *H. saturnus* (stable at pH 3–11, and 75% stable at 80 °C for 1 h). Similar results have been found for *Tilletiopsis albenscens* killer toxin, which is stable at pH 3.5–8 [12]. The genetic bases of killer characters of non-*Saccharomyces* yeasts are quite different from that of the K1 system. The killer toxin of *Kluyveromyces lactis* is encoded by linear dsDNA plasmids whereas *Pichia farinosa* killer toxin is chromosomally inherited [35]. Table 1 summarizes the distribution of killer toxins among yeast species and the genetic basis of some of the described killer toxins.

Diversity in the mode of action of killer toxins

The few toxins examined for action on *S. cerevisiae* appear to act like the K1 toxin in causing plasma membrane damage (Fig. 2). Toxins K1 and K2 are very similar in their mode of action; nevertheless, they are different proteins. A sequence of two reactions explains the activity of the K1 killer toxin of *S. cerevisiae*. The first step, the adsorption of the killer toxin to the cell-wall receptor (1→6)-β-D-glucan, is strongly pH-dependent [9, 27,28]. Although the cell-wall receptor is necessary for toxin action, it seems not to be the only component implicated in the killing process. At the following step, which is energy-dependent, the killer toxin interacts with a receptor on the plasma membrane, which results in the membrane becoming permeable to protons and potassium ions. These are the first distinct responses in sensitive cells, and are apparently due to the killer toxin acting as a K^+ ionophore or a protonophore. Later, the membrane becomes permeable to molecules of higher molecular mass such as ATP. Whether the killer toxin inhibits some component of the proton pump or acts more directly by forming a transmembrane protein channel remains unclear. The amphipathic character of the killer toxin proteins is consistent with the proposal that K1 killer toxin directly disrupts the transmembrane electrochemical gradients of the plasma membrane. Martinac et al. [18] showed that K1 toxin could form ion channels in vivo in sensitive spheroplasts, whereas it formed artificial liposomes in vitro, which suggests that these channels could kill sensitive yeast cells. The action of the killer toxin from *P. kluyveri* has been examined in detail [20]. This toxin is very similar to K1 in its action. After a lag of 50–90 min, physiological changes in the sensitive cells are observed in response to *P. kluyveri* killer toxin. Coordinated with potassium and ATP leakage, toxin-treated cells become more permeable to protons and intracellular pH drops, resulting in cell death. In our laboratory, we found similar results (unpublished) for a yeast strain isolated from olive brines and identified as *Pichia membranifaciens* CYC 1106, a killer strain with a broad spectrum of activity against other yeasts isolated from the same brines [15,17]. *P. membranifaciens* killer toxin is active against some sensitive strains such as *Candida boidinii* IGC 3430 only in the presence of sodium chloride [15]. This peculiarity has been studied to establish the role of NaCl in the killer character of this yeast. This killer toxin has an affinity to linear (1→6)-β-D-glucan [27], and this is the initial step for its toxic action. Cells of *C. boidinii* IGC 3430 treated with *P. membranifaciens* toxin had a lag of approximately 1 h before cell death. Metabolic events associated with the loss of *C. boidinii* viability were quantitatively identical to those known to occur with K1 killer-toxin-treated cells. Killing of sensitive cells of *C. boidinii* was characterized by potassium leakage, sodium influx and a drop in the intracellular pH (unpublished results).

The K28 killer toxin of *S. cerevisiae* differs from K1 and K2 killer toxins in that it is bound to the mannoprotein part of the yeast cell wall [38]; this feature has been used for the purification of K28. In contrast, K28 has no such ionophoric effect, but rather inhibits nuclear DNA synthesis [31]. K28-treated cells arrest in the budded phase of the cell cycle with an unreplicated (G1) content of DNA in a single nucleus located in the mother cell. Similar results have been found in *K. lactis* killer toxin. The action of this toxin is not immediate, since it must have been present continuously for more than 1 h before affecting a significant proportion of the treated cells. This toxin causes sensitive yeasts to arrest proliferation as unbudded cells, which suggests that it blocks completion of the G1 phase of the cell cycle [4].

The mechanism underlying the action of *H. mrakii* is not yet well understood. Its toxin kills sensitive strains presumably by interfering with β-(1→3)-glucan synthesis. Recent studies suggest, however, that a calcium-binding cell surface protein could be involved in the action of this killer toxin [36].

Applications

Killer yeasts and their toxins may have several applications. They have been used as model systems to study the mechanisms of regulation in eukaryotic polypeptide processing, secretion and toxin interaction with sensitive cells. Furthermore, the killer system in yeast provides useful models for the study of the control and expression

Table 1. Characteristics of the yeast killer toxins

Yeast species	Killer toxin	Subunits	Glyco-protein	Isoelectric point	Genetic basis	Primary receptor	Mechanism of killing	Application
Bullera sinensis	?	?	?	?	Chromosomal	?	?	?
Candida krusei	?	?	?	3.6–3.8	?	?	?	?
Candida glabrata	?	?	+	?	Chromosomal	?	Plasma membrane damage	?
Cryptococcus humicola	?	< 1 kDa	?	?	Chromosomal	?	?	?
Debaryomyces hansenii	?	23 kDa	?	?	Chromosomal	β-(1→6)-Glucan	?	?
Hanseniaspora uvarum	?	18 kDa	–	3.7–3.9	dsRNA	β-(1→6)-Glucan	?	?
Kluyveromyces fragilis	K6	42 kDa	?	?	?	?	?	?
Kluyveromyces lactis	?	α(97 kDa) β(31 kDa) γ(28 kDa)	?	?	dsDNA (pGKL1)	Chitin < ß2 >	Inhibition of cell cycle, G1 arrest	Avoid aerobic deterioration of silage
Kluyveromyces waltii	?	> 10 kDa	?	?	?	?	?	Control of *S. pombe* in wine making
Pichia acaciae	?	α(110 kDa) β(39 kDa) γ(38 kDa)	?	?	dsDNA (pPac1–2)	Chitin	?	Cell cycle arrest in G1, chitinase activity
Pichia anomala	?	83 kDa	?	?	?	?	?	Control of filamentous fungi in wood
Pichia farinosa	SMKT	α (6.3 kDa) β (7.7 kDa)	+	?	Chromosomal	?	Increase membrane permeability to ions	?
Pichia fermentans	?	?	?	3.8–4.2	?	?	?	?
Pichia inositovora	?	> 100 kDa	?	?	dsDNA (pPin1–3)	?	?	?
Pichia kluyveri	?	19 kDa	+	4.3	Chromosomal	?	Formation of ion channel	?
Pichia membranifaciens	?	18 kDa[a]	–	3.9[a]	Chromosomal[a]	β-(1→6)-Glucan	Formation of ion channel[a]	?
Saccharomyces cerevisiae	K1	α(9.5 kDa) β (9 kDa)	–	4.5	M_1-dsRNA	β-(1→6)-Glucan	Formation of ion channels, activation of K^+ channel	Avoid undesired contaminants in wine, beer, sake, etc. Genetics
S. cerevisiae	K2	$\alpha\beta$(21.5 kDa)	+	4.2–4.3	M_2-dsRNA	β-(1→6)-Glucan	Increase membrane permeability to ions	Wine fermentations
S. cerevisiae	KT28	α(10 kDa) β(11 kDa)	+	4.4	M_{28}-dsRNA	Manno-proteins	Entering into cell by endocytosis and inhibition of cell cycle, G2 arrest	?
Schwanniomyces occidentalis	?	α(7.4 kDa) β(4.9 kDa)	–	?	Chromosomal	Manno-proteins	Plasma membrane damage	?
Tilletiopsis albenscens	?	10 kDa	?	?	Chromosomal	?	?	?
Williopsis mrakii	HM-1	10.7 kDa	?	?	Chromosomal	β-(1→6)-, β-(1→3)-glucan	Inhibition of β-(1→3)-glucan synthesis	Silage, yogurt, taxonomy of *Nocardia*, Control of *C. albicans*
Williopsis saturnus	HYI	9,543 Da	–	5.8	Chromosomal	?	?	?
Zygosacch. bailii	KT412	10 kDa	–	4.1	dsRNA	Manno-proteins	?	?

[a]Unpublished results

of eukaryotic viruses. In addition, killer systems could have biotechnological applications both in fermentation industries and in medicine (Table 2). In recombinant DNA technology, killer plasmids from *S. cerevisiae* and *K. lactis* have the potential to serve as cloning vectors for the effective secretion of expressed polypeptides [39].

Fermentation

Killer yeasts are more abundant in natural habitats than in culture collections. The advantage that yeasts with killer characters have over sensitive strains in the environment can account for this abundance. Similarly, in the fermentation industries spoilage yeasts have high incidences of killer characters, which must also reflect their competitive advantage over commercial yeasts, most of which are sensitive to killer yeast strains [10, 29, 33,40]. In some fermentation processes, killer yeasts compete efficiently with brewing yeasts, this fact being of great importance in continuous culture. In batch cultures, there is competition only at high levels of contamination with the killer strain. At low levels, the large inoculum of the brewing yeast competes efficiently by consuming nutrients, which restricts the growth of contaminant killer strains. The optimum pH for production and stability of K1 killer toxin ranges from 4.6 to 4.8; therefore, it is not important for winemaking. However, K2 toxin, whose optimum pH ranges from 2.9 to 4.9, might pose a threat to the wine industry; K2 killer yeast causes protracted and blocked wine fermentation [40]. Pronounced killer activity occurs when the ratios of killer and sensitive cells are similar. To sum up, either neutral strains or killer strains having desirable oenological properties should be preferred. Killer yeasts could be used as starter cultures to control the growth of contaminant yeasts during the early stages of wine fermentation. To prevent spoilage by undesirable strains, killer yeasts suitable for brewing have been constructed by cytoduction [33]. The transfer of the killer character from one strain to another is possible without changing the nuclear genotype. Nuclear genotypes confer many significant biochemical features to industrial wine yeast strains. Protoplast fusion has been employed to obtain killer yeasts with good organoleptic properties [10].

Taxonomy

Yeasts make up a highly heterogeneous group of unicellular organisms. Although several discriminative criteria allow ascomycetous and basidiomycetous yeasts to be recognized, the distinction of different yeasts is often difficult. Molecular studies showed that many classical features used to define taxa, such as fermentation and assimilation of sugars, presence of spores, and morphology, are of limited value. At this point, the search for simple tests, which can be used widely, is of great importance. One of these tests could consist of finding out the differences in sensitivity to toxins produced by killer yeasts that are active against yeasts taxonomically related to killer-toxin producers [7,25]. According to the different sensitivities to killer toxins, it is possible to group yeasts into categories that are reproducible even if other characteristics are heterogeneous [6, 7, 21,25].

Medicine

Killer yeasts may have medical applications, particularly in the biotyping of the pathogenic yeasts *Candida albicans* and *C. neoformans*. The potential of these toxins as novel antimycotic agents for the treatment of infections has also been suggested [5,26]. Killer toxins are very labile at physiological temperatures and pH. However, their addition to buffered solutions might result in the effective treatment of yeast infections of the skin and mucosal membranes. Killer toxins cannot be used as systemic antibiotics because they are antigenic, as might be expected for large foreign proteins. It was possible, however, to obtain anti-idiotypic antibodies that exerted the same antimicrobial activities as the corresponding killer toxins. Polonelli et al. [26] produced these antibodies, which apparently shared the active site of the *P. anomala* toxin. These anti-idiotypic antibodies mimicked the killer effect of the secreted toxin against *C. albicans*.

Table 2. Potential applications of killer toxins

Biotechnological field of application	Application	References
Biological control in agriculture	Antifungal activity against wood-decay and plant pathogenic fungi. Prevention of aerobic spoilage of silage.	[11, 12,41]
Beverage fermentations	Avoid undesired contaminants in wine, beer, sake, etc.	[10, 24, 33,40]
Cellular biology research on eukaryotic cells	Studies of biosynthesis, cellular processing and secretion of proteins.	[30,43]
Food technology	Food preservatives of natural origin	[14,22]
Genetics	Selection of hybrids obtained by protoplast fusion; Fingerprinting of wine yeasts. Recombinant DNA technology (cloning vectors)	[10, 33,39]
Medicine	Zymocide activities against pathogens	[5, 26,32]
Taxonomy	Killer toxin sensitivity patterns may be indicative of phylogenetic affiliation.	[7, 13,25]

Conclusions

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 genetics, 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.

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