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Killer toxin of yeast: An overview

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Abstract
The first reports regarding their killer phenotype date to over 50 years ago, with the initial isolation of a Saccharomyces cerevisiae strain that inhibited the growth of other *S. cerevisiae* strains. Currently, the killer yeasts belonging to this species have been classified into three main groups (K1, K2, and K28) on the basis of the molecular characteristics of the secreted toxins, their killing profiles, the lack of cross-immunity, and the encoding genetic determinants. Killer strains of *Ustilago maydis* can secrete one of the three different toxins that have been identified so far. Many types of killer toxins have been reported and their genomes were mapped on double-stranded RNA (*S. cerevisiae* K1, K2, K28, *Ustilage maydis and Hanseniaspora uvarum*), a linear double-stranded DNA plasmid (*Kluyveromyces lactis, Pichia acaciae and Pichia inositovora*) or carried on a chromosome (S. cerevisiae KHS, KHR and *Williopsismrakii*). During the last two decades, secreted killer toxins and toxin-producing killer yeasts have found several applications. For instance in the food and fermentation industries, Killer yeasts have been used to combat contaminating wild-type yeasts which can occur during the production of wine, beer and bread. Killer yeasts have also been used as bio-control agents in the preservation of foods, in the bio-typing of medically important pathogenic yeasts and yeast-like fungi.

Keywords: Killer, toxin, *Ustilago maydis*, *Kluyveromyces lactis*

Introduction
The antagonistic activities of yeast against other microorganisms can be attributed to a number of different properties, including competition for nutrients and space, acidification of the medium, production of ethanol, and secretion of antimicrobial compounds, such as volatile acids, hydrogen peroxide, secondary metabolites, and the so-called killer toxins. For yeast, the first reports regarding their killer phenotype date to over 50 years ago, with the initial isolation of a Saccharomyces cerevisiae strain that inhibited the growth of other *S. cerevisiae* strains (Bevan and Makower, 1963; Woods and Bevan, 1968; Bussey,1972) [1, 25, 3]. According to these early investigations, killer (K) yeast secreted a toxin that was lethal to sensitive (S) strains of the same or related species, but was harmless to neutral (N) strains, which were immune to their killer effects. During the following years, the killer phenotype was shown to be widespread amongst yeast, with the description of almost 100 killer species that have been ascribed to about 46 genera (Klassen *et al*., 2017) [9]. Further studies have shown that the killer phenotype frequently occurs in yeast strains isolated from a variety of natural habitats (e.g. water, soil, fruit, and grape must) and from different geographic regions. Most killer yeast can kill other yeast of the same or of different species and genera. Some of them are active against filamentous fungi [Santos and Marquino,2004; Izgu *et al*., 2011) [22, 8] while others are also active against bacteria (Izgu *et al*., 2011; Meneghin *et al*., 2010; De Ullivari *et al*., 2014; Peerz *et al*., 2012) [8, 13, 5, 15].

Biodiversity of Killer toxin
The most well-characterized killer toxins with respect to their genetic determinants, biochemical characteristics, molecular targets on the sensitive cells, and mechanisms of killing are K1, K2, and K28 of *S. cerevisiae*, zymocin of *Kluyveromyces lactis*, PMKT and PMKT2 of *Pichia membranifaciens*, PaKT of *Wickerhamomycesanomalus*, HM-1 of *Cyberlindneramrakii*, and Kpkt of *Tetrapisisporaphaffii* (Santos and Marquino, 2004, Bussey *et al*., 1991 [21, 3].
For the remaining killer toxins, generally the localization of the genetic determinants of killer characters and the molecular weights of the killer toxins have been determined.

Although much still needs to be achieved regarding their modes of action and molecular targets within sensitive cells and for an understanding of toxin immunity. Despite this wide diversity, the killing action of all of the characterized killer toxins is generally mediated by a two-step mechanism. During the first step, the killer toxin recognizes and binds to a primary receptor on the cell wall of the sensitive target, thus indicating that the cell wall is an essential facilitator in the killing action. Accordingly, the killer toxins produced by *T. phaffi* (Kpkt), *Pichia anomala* (PiKT), and *Kluyveromycessiamensis* cannot kill spheroplasts at their sensitive targets [44-46]. Nevertheless, this is not a general rule, because PMKT and SMKT are also active on their target cells following enzymatic digestion of the cell wall (Santos *et al.*, 2007) [21]. However, it should be noted that the plasma membrane of yeast and fungi is negatively charged, while killer toxins can be more or less positively charged. Thus, the interactions between killer toxins and the plasma membrane of their sensitive target can also depend on the net cationic charge of the killer toxin. As the cell wall is in general the primary site of action of killer toxins, different cell-wall components can act as the primary receptor sites. Among these, the b-1, 3-D-glucans and b-1,6-D-glucans are commonly receptors for the majority of killer toxins characterized to date, although mannoproteins and chitin also serve as the first receptors for a number of killer toxins.

Classification of yeast killer phenomenon according to genetic basis

Cytoplasmic ally inherited encapsulated double-stranded RNA (dsRNA) viruses

Saccharomyces cerevisiae

The most thoroughly investigated yeast killer system is that of *S. cerevisiae*. Currently, the killer yeasts belonging to this species have been classified into three main groups (K1, K2, and K28) on the basis of the molecular characteristics of the secreted toxins, their killing profiles, the lack of cross-immunity, and the encoding genetic determinants. They are constituted by strains producing toxins encoded by double-stranded RNA (dsRNA), but other killer yeasts producing toxins named KHR and KHS, which are encoded on chromosome.

Ustilago maydis

Killer strains of *Ustilago maydis* can secrete one of the three different toxins that have been identified so far. These toxins, designated KP1, KP4, and KP6, have killer activity against susceptible cells of the same and closely related species (Koltin & Day, 1975) [10]. Each killer strain contains three to seven dsRNA segments, ranging in size from 0.36 to 6.2 kb, categorized into three groups: heavy (H), medium (M), and light (L). Six segments of dsRNA in P1 (H1 and H2, M1 to M3, and L), seven in P4 (H1 to H4, M2 and 3, and L), and five in P6 (H1, H2, M2, M3, and L) (Bozarth *et al*., 1981) [2].

Hanseniaspora uvarum, Phaffia rhodozyma* and *Zygosaccharomyces bailii

The killer phenotype associated with double strand RNA mycoviruses, similar to those in S. cerevisiae, have been detected in the yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii*. Four dsRNA molecules associated with virus-like particles, encoding a killer system, have been isolated from *Phaffia rhodozyma*. Their molecular sizes were approximately 4.3, 3.1, 0.9 and 0.75 kilobase pairs (kbp) as determined by agarose-gel electrophoresis and they were designated as L, M, S1 and S2, respectively (Radler *et al*., 1990, Radler *et al*., 1993, Castillo & Cifuentes, 1994) [18, 23, 4]. II-Linear double strand DNA plasmid *Kluyveromyces lactis* Killer strains always contain 50 to 100 copies per cell of each of two cytoplasmically inherited linear plasmids designated pGKL1 (k1) and pGKL2 (k2), which are 8.8 and 13.4 kbp in size, respectively.

K. lactis killer strains secrete a heterotrimeric toxin that inhibits the growth of a wide range of susceptible yeasts in the genera *Candida, Kluyveromyces, Saccharomyces, Torulopsis, and Zygosaccharomyces*, as well as non-killer strains of *K. lactis*. The toxin consists of three subunits: α polypeptide with a single asparagine-linked oligosaccharide unit, designated α (99kDa), and two smaller unglycosylated components, β (30 kDa) and γ (27.5 kDa). The precursor is targeted to the endoplasmic reticulum, where it is glycosylated, transported to the Golgi apparatus, and processed by a protease to form mature subunits. The mature toxin leads to the permanent arrest of susceptible cells in the unbudded (G1) phase of the cell cycle, in such a manner that they can never resume mitotic division. Despite previous reports, the toxin does not inhibit adenylate cyclase, but it causes a rapid and progressive loss of viability that is sufficient to explain the blockage of cell division (Sugisaki *et al*., 1984, Magliani *et al*., 1997) [24, 12].

Pichia inositovora

The presence of three linear dsDNA plasmids, of approximately 18, 13, and 10 kbp, has been reported in a killer toxin-producing strain of *P. inositovora*. Only two of them (pPin1-1 and pPin1-3) seem to be associated with the killer phenotype, while the loss of pPin1-2 has no effect on toxin production or susceptibility. The killer toxin apparently is an acidic heat-labile glycoprotein whose characterization and range of actions have not yet been determined (Magliani *et al*., 1997) [12].

Pichia acacia

P. acaciae killer strains have been shown to possess two linear plasmids, designated pPac1-1 (13.6 kbp) and pPac1-2 (6.8 kbp). These plasmids are quite similar in both function and structural organization to those found in *K. lactis*. Despite important similarities to *K. lactis* killer toxin, significant functional differences exist. *P. acaciae* toxin seems to be composed of three subunits (110, 39, and 38 kDa) with an associated chitinase activity. Chitin binding is essential to the activity of the toxin, which causes G1 cell cycle arrest. This toxin shows a wide range of activity, differing from but overlapping with that of *K. lactis*. All the linear plasmids so far identified, besides those associated with killer phenotypes, show similar promoter-like elements in their sequences, suggesting the existence of a unique but highly conserved expression system for these extra chromosomal elements.

Pichia anomala

The killer system of *P. anomala* has an activity against a wide range of unrelated microorganisms, such as yeasts, hyphomycetes, and bacteria, including important opportunistic pathogens such as *C. albicans* and the mycelial and yeast forms of the dimorphic fungi (Polonelli & Morace, 1986) [16]. A killer toxin, purified from a

strain (WC 65) of *Pichia anomala*, has been characterized and demonstrated to be an acidic glycoprotein of 83.3 kDa, stable between pH 2.0 and 5.0. Studies on the growth rates of a susceptible *C. albicans* strain in the presence of various toxin concentrations suggest the presence of two non-mutually exclusive binding sites for the toxin.

Pichia farinosa

A novel type of killer toxin produced by the halotolerant yeast *P. farinosa* has been recently described. This toxin, termed SMK (salt-mediated killer toxin), is a heterodimer (14.214 kDa), whose subunits (α, 6.6 kDa; β, 7.9 kDa) are tightly linked under acidic conditions. It shows its maximum killer activity in the presence of 2 M NaCl. Although there is no sequence similarity to other toxins, the 222-amino acid *P. farinosa* pre-protoxin resembles the *S. cerevisiae* K1 toxin in overall structure, hydrophobicity profile, and processing, suggesting that the target of the toxin is the membrane.

Pichia kluyver

P. kluyveri killer toxin,a 19-kDa acidic glycoprotein, induces the formation of ionpermeable channels, as does *S. cerevisiae* K1, which causes leakage of potassium ions and ATP, decrease of the cellular pH, and inhibition of amino acid uptake.

Pichia membranifaciens

Pichia membranifaciens CYC 1086 secretes a killer toxin (PMKT2) that is inhibitory to a variety of spoilage yeasts and fungi of agronomical interest. The killer toxin in the culture supernatant was concentrated by ultrafiltration and purified to homogeneity by two successive steps. Biochemical characterization of the toxin showed it to be a protein with an apparent molecular mass of 30kDa and an isoelectric point of 3.7. At pH 4.5, optimal killer activity was observed at temperatures up to 20 ºC. Above approximately this pH, activity decreased sharply and was barely noticeable at pH 6. Strains of *P. membranifaciens* produced at least two different types of toxins, named PMKT and PMKT2. PMKT2 had physico-chemical properties and molecular mass similar to PMKT, but their spectra of biological activity against a variety of fungal and yeast strains were different, indicating that they were different toxins.

Schwanniomyces occidentalis

The yeast *Schwanniomyces occidentalis* produces a killer toxin lethal to sensitive strains of Saccharomyces cerevisiae. Killer activity is lost after pepsin and papain treatment, suggesting that the toxin is a protein. The killer protein was composed of two subunits with molecular masses of approximately 7.4 and 4.9 kDa, respectively. Maximum killer activity was between pH 4.2 and 4.8. The protein was stable between pH 2.0 and 5.0 and inactivated at temperatures above 40 °C. The killer protein was chromosomally encoded. Mannan, but not β-glucan or laminarin, prevented sensitive yeast cells from being killed by the killer protein, suggesting that mannan may bind to the killer protein.

Applications

Food and fermentation industries

The food and beverage industries were among the first to explore the application of killer – toxin producing yeasts to kill spoilage microorganisms (Lowes *et al*., 2000) [11]. Yeast strains often achieve competitive advantage by producing killer toxins, which kill off competing species sensitive cells belonging to either the same or a different species (Santos *et al*., 2009) [22].

II- Killer yeast as potential antimicrobial agents

The finding that the killer activity could be displayed against a great variety of eukaryotic and prokaryotic microorganisms led to a re-evaluation of the yeast killer phenomena, with special emphasis on the surprising susceptibility of microorganisms of clinical interest such as *Candida albicans, Pneumocystis carinii and Mycobacterium tuberculosis* (Magliani *et al*., 1997) [12].

As antifungal agent

Antifungal research is currently focusing on the possible use of yeast killer toxins as novel antifungal agent (Schmitt & Breinig, 2002) [23]. Killer toxins in future might find application in the treatment of fungal infection (Magliani *et al*., 1997) [12]. Within this group, secreted killer toxins mainly produced by non-Saccharomyces yeasts show a broad spectrum of killing activity against a great number of human and plant pathogens (Schmitt & Radler, 1988) [23].

As antibacterial agent

Killer activity of yeast might operate over bacteria and could be used for the bio- control of contaminating bacteria for alcoholic fermentation. (Polonelli & Morace, 1986, Meneghin *et al*., 2010) [13]. It was reported that toxins from *C. glabrata, P. anomala and T. figueirae* were found to be active against *Lactobacillus plantarum* and *Bacillus subtilis* (Polonelli & Morace 1986) [16].

III- Yeast killer system in bio-typing

Killer system may be effective and inexpensive tool for yeast finger printing and could be used for intraspecific characterization of industrially and clinically interesting yeast cultures. The first application of the yeast killer system for intraspecific differentiation of pathogenic fungi was reported for *Candida albicans* isolates. The killer system has proven to be fruitful not only in differentiation of important slowly growing pathogenic, such as the mycobacteria, but also in the differentiation of faster-growing Gram positive (Izgu *et al.*, 1997) [8] and Gram negative bacteria (Polonelli *et al*., 1987) [17].

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