CLINICAL MICROBIOLOGY REVIEWS, July 1997, p. 369–400 Vol. 10, No. 3
0893-8512/97/$04.00+0

Yeast Killer Systems

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BIOLOGY OF YEAST KILLER SYSTEMS 369
Yeast Killer Phenomenon 369
***Saccharomyces cerevisiae* Killer System 370**
L-A mycoviruses 370
Satellite M dsRNAs 371
K1, K2, and K28 toxins 371
Mode of action of K1, K2, and K28 toxins and self-immunity systems 373
Yeast cell-virus interaction and killer-sensitive relationships 373
KHR and KHS killer toxins 374
***Ustilago maydis* Killer System 374**
***Kluyveromyces lactis* Killer System 375**
***Pichia* and *Williopsis* Killer Systems 377**
Other Yeast Killer Systems 380
ECOLOGY OF YEAST KILLER SYSTEMS 380
Environmental Yeast Killer Systems 380
Industrial Yeast Killer Systems 382
Opportunistic Killer Yeasts 383
In Vivo Production of Yeast Killer Toxins 383
Experimental Enterotoxicity of Yeast Killer Toxins 384
Interferon Induction by dsRNA Yeast Killer Plasmids 384
EPIDEMIOLOGY BY YEAST KILLER SYSTEMS 384
Reevaluation of the Yeast Killer Phenomenon 384
Strain Differentiation by the Yeast Killer System 385
Species and Variety Identification by the Yeast Killer System 386
THERAPEUTIC POTENTIAL OF YEAST KILLER SYSTEMS 386
Antibiotic Properties of Yeast Killer Toxins 386
Therapeutic Properties of Yeast Killer Toxins 387
SEROLOGY OF YEAST KILLER SYSTEMS 387
Antigenicity of Yeast Killer Toxins 387
Monoclonal Antibodies against Yeast Killer Toxins 387
Serological Analysis of Yeast Killer Toxins 387
IDIOTYPY OF YEAST KILLER SYSTEMS 388
Origin of the Antibody Repertoire 388
Idiotypic Network 388
Anti-Idiotypic Vaccination 388
Yeast Killer Toxin Anti-Idiotypes 389
Idiotypic Vaccination 390
Natural Human Yeast Killer Toxin-Like Antibodies 390
Perspectives 391
REFERENCES 392

BIOLOGY OF YEAST KILLER SYSTEMS

Yeast Killer Phenomenon

The production by yeasts of exotoxins with antimicrobial activity mediated by specific cell wall receptors on susceptible microorganisms is a relatively common phenomenon (288, 298, 356, 432, 453, 455). Exotoxins (generally proteins or glycoproteins) that are able to kill susceptible cells belonging to the same or congeneric species have been defined as killer toxins. Killer yeasts are toxin-producing fungi that are immune to the activity of their own killer toxins. After the original description of the phenomenon in *Saccharomyces cerevisiae* reported by Bevan and Makower in 1963 (18), the attention of an increasing number of investigators has been focused on the killer effect, which might represent a model of biological competition somewhat related to that of bacteriocins among bacteria (158). Related studies have proved to be of great value in clarifying the molecular characteristics of the various killer toxins, their physiology, their mode of action, and the genetic determinants responsible for their production. These studies, moreover, have been important for examining certain aspects of the struc-

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ture and functions of eukaryotic cells because fungal cells, and yeasts in particular, are an attractive model for studying the mechanisms of processing and secretion of extracellular proteins. Their secretory pathway is similar to that found in higher organisms (264). The study of the yeast killer toxin receptors of susceptible microorganisms and resistant mutant strains has contributed enormously to the knowledge of the structure and function of the yeast cell wall. This wall is no longer considered a mere envelope providing only physical and osmotic protection to a cell but, rather, is now known to be a dynamic cell component with essential functions. It is a complex structure that provides selective permeability, enzyme support, and cell-to-cell recognition and adhesion and plays a role in the protein secretory pathway (370).

Studies of mycoviruses have given an insight, at least in part, to the relationships between the virus and the yeast cell in a system remarkably easier and simpler to study than the system consisting of a virus and an animal cell; they have also allowed the definition, even though incompletely, of which gene products are involved in the infection.

The newest technologies of molecular biology and genetic engineering have allowed the construction of plasmid vectors by using nucleotide sequences of viruses and plasmids involved in the synthesis and secretion of yeast killer toxins. These vectors have been used for the expression, secretion, and production of heterologous proteins in yeasts (22, 340).

After the first studies on the nature of the killer factor showed the involvement of cytoplasmic non-Mendelian genetic determinants, the occurrence in killer yeasts of double-stranded RNA (dsRNA) associated with virus-like particles was proposed (3, 15, 19, 20, 166, 356, 415, 416, 441). Since then, the yeast killer phenomenon has been discovered in a great number of yeast genera and species (280, 432, 455). It is associated with the presence of viral dsRNA, linear DNA plasmids, or nuclear genes in some yeasts, but the genetic basis of other killer systems remains unknown. Curing of the killer plasmids by using heat shock, UV irradiation, ethidium bromide, cycloheximide, acridine orange, 5-fluorouracil, or ethyl methanesulfonate, as well as the isolation of spontaneously arising nonkiller strains, confirmed that either dsRNAs or plasmids were responsible for killer activity. Conversely, the introduction of the killer gene determinants from killer yeasts into susceptible nonkiller strains conferred on the recipients the killer inheritable character (58, 64, 111, 147, 162, 262, 308, 336, 426, 429, 442). Besides *S. cerevisiae*, in which at least three different killer systems have been described, three other systems have been reported in the smut fungus, *Ustilago maydis*, and one has been found in *Kluyveromyces lactis*. Killer strains have been found also in some species of a variety of other yeast genera, such as *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Torulopsis*, *Williopsis*, and others, including yeasts of environmental, industrial, and clinical interest.

Following initial observations, which limited the activity of killer toxins to restricted species of yeasts, the finding that the killer activity could be displayed against a great variety of unrelated eukaryotic and prokaryotic microorganisms led to a reevaluation of the yeast killer phenomenon, with special emphasis on the surprising susceptibility of microorganisms of clinical interest such as *Candida albicans*, *Pneumocystis carinii*, and *Mycobacterium tuberculosis* (5, 253, 286, 288).

One of the most outstanding aspects of the phenomenon which has become apparent in the past few years has been the immunological implications based on the theory of the idiotypic network proposed by Jerne in 1974 (183). Antibody derivatives of a *Pichia anomala* killer toxin have been extensively studied to evaluate their potential use as idiotypic vaccines or anti-idiotypic antibiotics for a conceptually new type of antifungal immunotherapy. It has been intriguing to verify that apparently new ways of antifungal prevention and control have been adopted by the immune system in the course of natural infections.

Saccharomyces cerevisiae Killer System

The most thoroughly investigated yeast killer system is that of *S. cerevisiae*, which has been described in detail in many reviews (42, 51, 135, 376, 395, 396, 432, 433, 435, 437). 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 dsRNA, but other killer yeasts producing toxins named KHR and KHS, which are encoded on chromosomal DNA, have been defined (142–144). Study of the K1 *S. cerevisiae* system, in particular, has led to extensive information on mycoviruses, their relationship to the host yeast cell, and the physiological and structural characteristics of eukaryotic fungal cells.

The K1, K2, and K28 toxins are encoded by different cytoplasmically inherited satellite dsRNAs (M1, M2, and M28), encapsidated in virus-like particles (VLPs) and dependent on another group of helper yeast viruses (L-A) for their replication and encapsidation. The M dsRNAs are responsible for either killer activity or self-immunity, a phenotype that is characteristic of yeast killer toxin-producing strains. The toxins are able to kill nonkiller yeasts as well as yeasts of the opposite killer class, while the producing yeasts remain immune to their own toxin and to that produced by strains of the same killer group.

As stated above, killer systems provide a unique model for study since, in the killer strains, infection with L-A virus is associated with the presence of a satellite M dsRNA, packaged in a capsid encoded by the helper virus. L-As are autonomously replicating viruses that do not require M RNA for replication.

L-A mycoviruses. The presence of viruses with dsRNA genomes in fungi has been known for a long time, even though, with a few exceptions, their biological significance remains unknown. Recently, the dsRNA mycoviruses have been classified in two families, *Totiviridae* and *Partitiviridae*. The genus *Totivirus*, consisting of L-A helper viruses, belongs to the former family (43, 44). L-A viruses are 39-nm-diameter isometric particles with no envelope, containing an undivided dsRNA genome whose complete nucleotide sequence (4,579 bp) has been determined (83, 99, 176).

Recently, the genome organization, replication strategy, virion assembly, and biological properties of L-A viruses were extensively reviewed (135, 437). Mature L-A virus particles contain one 4.6-kb dsRNA molecule and have an associated transcriptase activity that synthesizes in vitro positive-strand RNA containing an unpaired A residue at its 3′ terminus (4,580 bases) (42, 99, 117, 176). The positive-strand RNA is extruded from the virus particles on synthesis and serves as the mRNA for synthesis of the viral proteins, as the nucleic acid encapsidated to make new viral particles, and as the template for viral replication (99, 118). This RNA contains two open reading frames (ORFs), a virus binding site (VBS) 400 bases upstream of the 3′ terminus, and an internal replication enhancer, partially overlapping with the VBS, which is necessary for full template activity of the RNA. As in retroviruses, the 5′ ORF (ORF1; 2,043 bases) encodes the major coat protein (called Gag; 76 kDa), while the 3′ ORF2 (Pol), overlapping

with ORF1 by 130 nucleotides, is expressed only as a 180-kDa Gag-Pol fusion protein which is formed by a −1 ribosomal frameshift (85, 104, 120, 121, 176). The C-terminal domain of the fusion protein (Pol) has single-stranded-RNA-binding activity (119). It includes the consensus amino acid sequence pattern for RNA-dependent RNA polymerases typical of positive-strand RNA and dsRNA viruses (313).

The replication of L-A dsRNA is similar to that of the reoviruses, occurring in a sequential manner by a conservative mechanism (117, 259, 260, 342). Positive strands are synthesized by the virion-associated RNA-dependent RNA polymerase inside the viral particles by end-to-end transcription of dsRNA. They are then extruded from the particles and translated to make viral proteins (99). The Pol domain of the Gag-Pol fusion protein binds specifically to the VBS on positive strands, and the Gag domain primes the polymerization of free Gag to form the capsid, thus ensuring the packaging of both the viral genome and the RNA polymerase. Some of the positive-strand RNAs become encapsidated in the new viral particles and serve as the templates for the synthesis of negative strands to produce dsRNA inside the virus-like particles (117, 120, 122). L-A mycoviruses can support the replication and encapsidation of one of several satellite M dsRNA, each of which encodes a killer-immune system. The study of the interactions of L-A with the M satellite genomes has permitted the identification of at least four natural variants of L-A dsRNA (L-A-H, L-A-E, L-A-HN, and L-A-HNB). Each carries different combinations of genetic activities, called *HOK* or helper of killer (H), *NEX* or nonexcluder of M2 (N), *EXL* or excluder of M2 (E), and bypassing *MAK* (B) mutations, according to their ability to maintain or exclude M1 and M2 dsRNAs of different genetic backgrounds (395, 407, 433). It has been recently demonstrated that in the K28 killer system, L28 is an L-A-H species with *HOK* activity lacking B activity (336).

Satellite M dsRNAs. Satellite M dsRNAs are a family of RNA molecules present in VLPs persisting in the cytoplasm of *S. cerevisiae* killer strains. They are dependent on L-A helper viruses for their replication and encapsidation (31, 94, 155, 336, 395). 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, i.e., for the production and secretion of killer toxins to which these strains are immune or resistant but which are lethal to susceptible strains of the same or different yeast species. All three killer toxins of the best-known killer strains (K1, K2, and K28) are encoded by different dsRNAs (M1, M2, and M28), differing in size (1.8, 1.5, and 1.9 kb, respectively) and showing similar organization, even without any significant sequence homology (84, 135, 335, 336, 339, 435, 437).

Maintenance of M dsRNA is dependent on the expression of both ORFs of L-A, and it has been observed that essentially all L-A variants and M dsRNAs are interchangeable. However, L-A-HN and L-A-H are the variants found in all K1 or K2 wild-type killer strains, respectively. Independent of genotype, M1 excludes M2 from any killer strain (135, 431).

The replication cycle of M dsRNA depends on L-A functions and is similar to that of the helper virus, occurring conservatively within the virions but with some differences (439). M dsRNA is less than half the size of L-A dsRNA and is packaged in L-A-encoded capsids that can accommodate one molecule of the larger dsRNA. As a consequence, M dsRNA shows a "headful replication" mechanism: the positive-strand RNA transcripts are not extruded, initially, but they are retained within the particle, serving as a template for negative-strand synthesis to form a second dsRNA molecule. Positive-strand RNAs are released only when the particle is full, completing the replication cycle (99).

Both M1 and M2 RNAs contain an internal AU-rich "bubble," 130 to 200 nt long, separating a single 5′ toxin-encoding ORF and a 3′ noncoding region involved in essential functions for replication and encapsidation (155, 246, 395). The M28 dsRNA seemed to lack such a region, since oligo$(dT)_{15}$ could not be used to prime cDNA synthesis (335). Recently it has been shown, however, that the M28 positive strand and the M28 positive-strand RNA in vitro transcript can bind to oligo(dT)-cellulose columns, indicating the presence in the positive strands of an internal A-rich region of at least 40 A residues (339). Sequencing of the 5′ and 3′ regions of the three positive-strand RNAs showed that a striking homology exists only at the very end of 5′, with a consensus sequence of 6 bases possibly involved in the initiation of positive-strand RNA synthesis (41). The 5′ end of M1, M2, and M28 positive-strand RNAs contains the AUG initiating codons at positions 14 to 16, 7 to 9, and 13 to 15, respectively, representing the beginning of each killer toxin ORF. These 5′-terminal regions contain similar strong secondary stem-loop structures that enclose the initiating codons and show some complementarity to the 3′ termini of 18S and 5.8S rRNA molecules, and are probably involved in their translation processes (222).

Most knowledge of these killer systems has been obtained by cloning cDNAs and expressing them in susceptible nonkiller strains of *S. cerevisiae*, thus allowing studies on the mechanisms of toxin production, activity, and immunity (33, 84, 154, 228, 246, 339, 354). In all three systems, the expression of the cDNA directs the production of killer toxins and immune components with the same specificity as the native dsRNAs, showing that killing and immunity functions are coded for by the same respective ORFs (Fig. 1).

K1, K2, and K28 toxins. K1, K2, and K28 *S. cerevisiae* killer toxins are protein molecules secreted by killer strains carrying the specific satellite dsRNA; killer strains are not susceptible (are immune) to their own toxin but remain susceptible to other killer toxins. Even though the toxins have different amino acid compositions and modes of molecular action, they show some general characteristics in their mechanisms of synthesis, processing, and secretion. Each toxin is encoded by a single ORF and synthesized as a single polypeptide preprotoxin comprising larger hydrophobic amino termini than are usually found on secreted proteins and potential kex 2/kex 1 cleavage and N-linked glycosylation sites; the preprotoxins have similar overall structures. The preprotoxins, once synthesized, undergo posttranslational modifications via the endoplasmic reticulum, Golgi apparatus, and secretory vesicles, resulting in the secretion of the mature, active toxin.

The best-studied and best-known killer toxin, K1 (19 kDa), is secreted as a molecule consisting of two distinct disulfide-bonded unglycosylated subunits, termed α (9.5 kDa) and β (9.0 kDa), derived from a 42-kDa glycosylated precursor molecule (protoxin). The primary translation product of M1 dsRNA is a 316-amino-acid, 35-kDa polypeptide (preprotoxin, M1p) consisting of a 44-amino-acid N-terminal leader sequence called δ, which includes a 26-residue signal peptide, followed by the 103-residue α domain (positions 45 to 147) and the 83-residue β domain (positions 234 to 316) of toxin subunits, which are separated by a central γ peptide (positions 148 to 233) carrying all three potential N-glycosylation sites (33, 354, 375, 456). Once synthesized, the preprotoxin enters the endoplasmic reticulum, directed by the leader sequence or some part of it, and the signal peptide is removed by a peptidase that probably cleaves at $ValAla^{26}$ to produce protoxin (229). The remaining 27- to 44-amino-acid segment is presumably removed in the

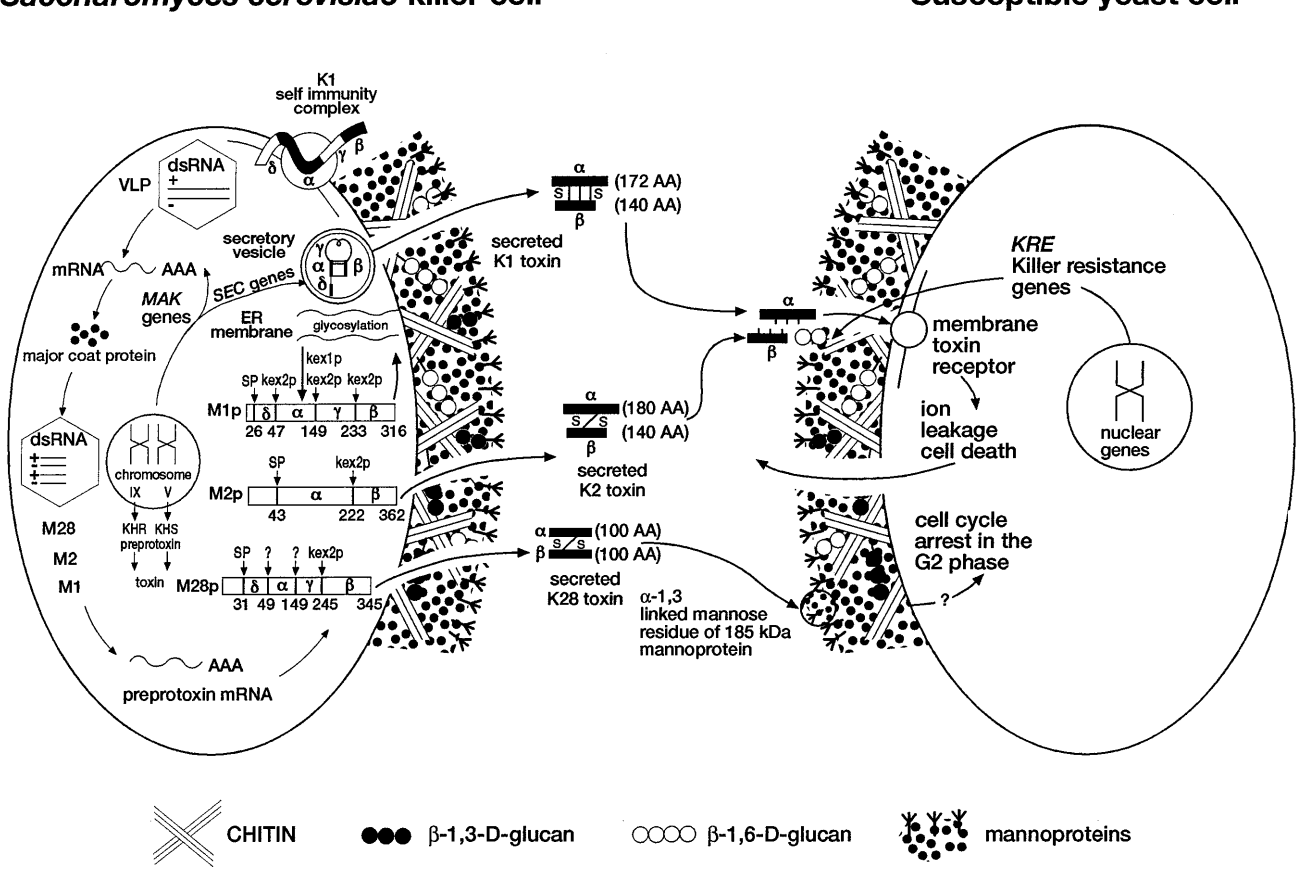

FIG. 1. Sketch of the genetics, toxigenesis, immunity, and activity of the *S. cerevisiae* killer system. AA, amino acids.

Golgi apparatus by an unidentified protease present in yeasts, which cleaves at ProArg[44] (34, 459). In the endoplasmic reticulum, the γ domain is N glycosylated and presumably folds into a form competent for translocation to the Golgi and for further processing, by a mechanism involving at least three proteolytic cleavages (32, 50). An endopeptidase encoded by the yeast *KEX2* gene (kex2p) is responsible for the cleavages which follow pairs of dibasic residues (ArgArg[149], LysArg[188], and LysArg[233]) and involve the termini of the α domain and the amino terminus of the β domain to generate both subunits (187, 250). These cleavages presumably occur at different rates or with different efficiencies in the same vesicle in a late Golgi compartment, probably to minimize the production of lethal fragments or to maximize the production of fragments involved in the expression of immunity (459). The α subunit is further processed by a serine carboxypeptidase, encoded by the yeast *KEX1* gene (kex1p), which removes dibasic C-terminal residues (88). Finally, the mature toxin is secreted outside the cell via the established yeast secretory pathway as a dimeric molecule in which the subunits are covalently linked by disulfide bonds that probably link the three cysteine residues in α and β. The study of the K1 toxin maturation process has been extremely fruitful and has led to an understanding of most of the mechanisms involved in the processing and secretion of proteins, such as peptide hormones, in higher eukaryotes and to knowledge of the role of the kex proteases or their homologs in all eukaryotes (38, 125).

The K2 and K28 toxins have been characterized more recently and less extensively than K1, but they seem to show a similar overall organization, mainly at the precursor level. K2 is synthesized as a 362-amino-acid precursor of 38.7 kDa (M2p) containing three potential sites for Asn-linked glycosylation at amino acid residues 177, 214, and 261; a potential N-terminal secretion signal; and potential kex1p and kex2p cleavage sites (84, 246). During the maturation process, the signal peptide is removed by peptidase cleavage after Ala[43] and the remaining molecule apparently is cleaved by the kex2p after Arg[222], yielding the two subunits (α and β) that constitute the mature secreted toxin. Unlike the K1 toxin, a γ domain does not seem to be present in the preprotoxin. The final α and β subunits are larger than those of K1 (172 and 140 amino acids, respectively), and α is N glycosylated at two positions (positions 177 and 214). Finally, kex1p is also required for the complete processing of α.

The recently published complete sequence of the M28 dsRNA has emphasized the similarity of preprotoxin synthesis to that of K1 (339). K28 toxin, previously thought to be a serine-rich monomeric 16-kDa glycoprotein carrying O-linked carbohydrate, is synthesized as a 345-amino-acid preprotoxin (M28p; 37.6 kDa) beginning with a potentially redundant pair of secretion signal peptides that are probably removed by peptidase cleavage after Leu[31] at the entrance to the endoplasmic reticulum (278, 335). The toxin is secreted as a disulfide-bonded heterodimer of α (10.5 kDa) and β (11 kDa) subunits, separated in the preprotoxin molecule by a γ domain (residues 149 to 245) in which the three N-glycosylation sites are located. Whereas a kex2p site occurs before the β amino terminus (residue 245), making this endoprotease essential in the pro-

cessing of the toxin, the cleavage sites of the α C terminus and the enzyme involved have not been determined, although Arg^{149} might be the most probable cleavage site. Kex1p is also important for toxin activity, presumably intervening in the final processing of either the α or β C-termini.

Mode of action of K1, K2, and K28 toxins and self-immunity systems. All the secreted mature toxins can exert killer activity on susceptible cells by different mechanisms that require a specific initial binding to a cell wall receptor, the precise structure of which remains largely unknown. Once again, the mode of action of the K1 killer toxin has been the most extensively investigated. Recent kinetic studies indicate that the susceptible cells contain two populations of toxin-binding sites that interact with the killer toxin with widely different affinities (215). The first step of the binding is strongly pH dependent with an optimum at pH 4.6 and is a low-affinity, high-velocity adsorption (1 min) of the killer toxin to the cell wall receptors, which are present at an average of 1.1×10^7 molecules per cell (48). The second step is a high-affinity, low-velocity, energy-dependent interaction of the toxin with a probable plasma membrane receptor that leads to the actual lethal effect (457). The constituents of the glucan fraction of the cell wall, mainly β-1,6-D-glucan, have been identified as primary receptors for the toxin, and their assembly seems to require a number of yeast *KRE* (killer resistance) genes (4, 30, 39, 168, 174). Most receptors appear unnecessary for toxin activity because only a few glucan receptors in close proximity to the plasma membrane, i.e., at the site of final assembly of glucan at the bud tip, seem to be functional. This could explain why young budding cells are the most susceptible to the toxin (51). Both subunits of the mature toxin seem to be necessary for receptor binding, with the hydrophilic β subunit being primarily responsible for the binding and the α subunit acting in a multifunctional way, with different overlapping regions of the polypeptide involved in killer activity, immunity, and binding (458).

After binding to the yeast cell wall, K1 toxin is transferred to the cytoplasmic membrane and acts by forming voltage-independent cation transmembrane channels, which cause ion leakage and subsequent cell death (79, 236). Two strongly hydrophobic regions near the C terminus of the α subunit (residues 72 to 91 and 112 to 130) have an α-helical structure separated by a short, highly hydrophilic segment and may act as a membrane-spanning domain responsible for channel formation (375).

K2 has virtually identical toxin activity to that of K1, despite a different structure, but K28 seems to act on the cell cycle in a different way. The K28 toxin binds primarily to the α-1,3-linked mannose residues of a 185-kDa cell wall mannoprotein, causing cell cycle arrest, apparently in the G_2 phase, and leading to nonseparation of mother and daughter cells, with the nuclear DNA confined to the mother cell. It is not clear if this early reversible inhibition of DNA synthesis is a primary or secondary effect of the toxin (332–335).

Susceptible strains can become resistant to a killer toxin by a mutation in any 1 of the 11 *KRE* genes (*kre* mutants) so far identified that are involved in cell wall receptor synthesis. Their spheroplasts, however, remain susceptible to toxin, implying the existence of a secondary membrane receptor (30, 244). Yeast killer strains, on the other hand, are not susceptible (are immune) to the toxin they produce, bind normal amounts of toxin to their cell wall, and have resistant spheroplasts, probably because the specific membrane receptor is masked, modified, or destroyed. The killer-immune phenotype is determined by the M dsRNA and cDNA copies of these RNAs (84, 154, 228, 246, 339, 396). By site-directed mutagenesis of the preprotoxin gene and studies of genetic mutations in the host cell, it has been possible to demonstrate that the precursor of the toxin is responsible for host cell immunity in K1 strains (28, 51, 375, 458, 460). The exact mechanism of this phenomenon is not yet clearly understood, but the α and γ components of the precursor, even in the absence of processing, seem to be required for the expression of immunity. The immunity determinant appears essentially as an extension of the toxic domain of α, involving the central region of α and the N terminus of γ (460). The γ domain seems to play a critical role in promoting the functional maturation of protoxin, probably acting as an internal chaperone for protoxin folding into a form competent for translocation from the endoplasmic reticulum to the Golgi apparatus and for the final processing by kex2p and kex1p. The protoxin or its fragments probably interact with a membrane receptor, masking or modifying it, before the production of the mature toxin. The 31 N-terminal residues of γ probably interact specifically with α, blocking its toxic activity but not its interaction with the receptor, until the final processing and secretion of the toxin. The K2 and K28 strains show a similar immune phenomenon, although in K2, immunity and toxic activity appear independent (84).

Yeast cell-virus interaction and killer-sensitive relationships. L-A viruses and the associated satellite dsRNAs can be transmitted intracellularly only during cell division, sporogenesis, or cell fusion; the rigid cell wall of the fungi constitute a barrier to virus uptake. Efficient transfection has been obtained in vitro by inoculating spheroplasts with virus particles from K1, K2, or K28 killer strains (95, 335).

The host plays a critical role in the maintenance and expression of the killer phenotype. The killer systems provide an excellent model for studying the chromosomal genes involved in the phenomenon and their role for the host. As extensively reviewed recently by Wickner (436), besides the *KEX1* and *KEX2* genes, which encode proteases necessary for processing the protoxins as well as other yeast preproteins, other yeast genes (*SEC* genes) are involved in the secretion of the mature toxin (50, 90, 229, 459).

Two groups of host genes can affect the propagation of L-A and M1: the superkiller (*SKI*) genes and the maintenance of killer (*MAK*) genes. The products of the *SKI2* to *SKI8* genes, named for the phenotype of the mutants, repress the copy number of M1 and the translation of its mRNA. Ski2, a large protein of the helicase family, has also been shown to repress L-A, L-BC, and the 20S ssRNA replicon (10, 239, 397). The *SKI* genes appear to constitute a host antiviral system that is essential to the cell only for repressing viral propagation (240, 312, 357, 438). Recently, *SKI2*, *SKI3*, and *SKI8* have been shown to act by specifically repressing the translation of non-poly(A)$^+$, 5′–uncapped mRNAs, such as those of L-A and M1 (237).

More than 30 chromosomal *MAK* genes, important or essential for cell growth, are necessary for propagation and maintenance of the killer phenotype. Only three of them (*MAK3*, *MAK10*, and *PET18*) are needed for the propagation of L-A dsRNA. The *MAK3* gene encodes an *N*-acetyltransferase, which is probably involved in N-acetylation of mitochondrial proteins and is responsible for the acetylation of the N terminus of the major coat protein required by L-A and M1 for viral assembly (392, 393). The *MAK10* product, a protein needed for optimal growth of the fungus on nonfermentable carbon sources, is similar to the α subunits of T-cell receptors, is required by both L-A and M for their propagation, and probably stabilizes the complete viral particle (220). The *PET18* gene product, which is needed for replication of mitochondrial DNA and for cell growth, is probably particle associated and

contributes to the stability of the virions, as does the *MAK10* product (117, 118).

In addition to the L-A-encoded proteins, many more *MAK* genes are necessary for the propagation of the killer-encoding M satellite dsRNA. These gene products include different 60S ribosomal subunit proteins, such as L3 (*MAK8*), L4A (*MAK7*), L4B (*KRB1*), and L41B (*MAK18*); a DNA topoisomerase I (*MAK1*); an essential membrane-associated protein with β-transducin repeats (*MAK11*); and a nuclear protein required to transit G_1 (*MAK16*) (62, 175, 265, 266, 341, 394, 430, 434, 435). Most of the *mak* mutations result in a decrease in the number of free 60S subunits or in poor association of 60S and 40S subunits (265). It has been suggested that L-A may provide M1 with Gag and Gag-Pol only after its own protein requirement has been met (preferential *cis* packaging) (86, 87, 265, 409). By reducing the efficiency of L-A positive-strand translation and thus the supply of proteins from the helper virus, any *mak* mutation that decreases the concentration of the 60S subunit could result in the selective loss of M1.

Susceptibility to killer toxins requires a number of functional host genes (*KRE*) involved in the expression and assembly of the specific receptors, mainly in glucan or mannan synthesis (51, 332). By studying mutants resistant to K1 toxin, at least seven genes implicated in cell wall synthesis have been identified. The *KRE1* and *KRE5* genes encode secretory pathway proteins involved in β-1,6-D-glucan synthesis (30, 244). The *KRE6* and *KRE2* gene products are a probable membrane-associated glucan synthase responsible for the production of normal levels of glucan and a mannosyl transferase required for the cross-linking of glucans with mannoproteins in the cell wall, respectively (161, 168, 315, 316). Other genes involved in the glucan biosynthetic pathway, like *KRE9 KRE10*, *KRE11*, and *CHW41*, have been identified recently (39, 184). The study of these genes and their products has been invaluable for exploring and understanding the complex mechanisms involved in the synthesis and assembly of the cell wall of yeasts. The search for other genes will enhance our current knowledge. Molecular and genetic studies suggest that mature β-1,6-D-glucan is formed by the synthesis of a core glucan from UDP-glucose, involving kre5p, kre6p, and kre11p, followed by kre1p-dependent side chain addition (370). Spheroplasts of these *kre* mutants, as well as the spheroplasts of a wide range of other yeasts, are still susceptible to the toxin, suggesting a common initial mechanism of adsorption to the plasma membrane (457). *kre3* mutants are not susceptible to the toxin but still bind normal amounts of it to the cell wall, suggesting a mutation in a nuclear gene involved with the plasma membrane receptor synthesis (4, 90). A chromosomal gene (*KRE*12) has been identified in *kre* mutants that were resistant to the toxin as spheroplasts. The gene product probably represents a secondary K1 membrane receptor which acts as a docking protein, allowing toxin binding to the membrane and ion channel formation (338).

KHR and KHS killer toxins. As mentioned above, other killer toxins have been described in *S. cerevisiae*, and two of them, which are chromosomally encoded, have been designated KHR (killer of heat resistant) and KHS (killer of heat susceptible), because they differ in their thermostability and optimum pH. The mature KHR and KHS toxins are single proteins, with molecular masses of about 20 and 75 kDa, encoded on the left arm of chromosome IX and on the right arm of chromosome V, respectively (142–144). Since the mature toxins have molecular masses lower than those of their precursors, some protein processing is thought to occur during maturation. The *KHR* and *KHS* genes consist of 888 and 2,124 bp, respectively, with no homology to other killer genes.

KHR encodes a preprotoxin of 33 kDa, which has a possible hydrophobic signal sequence in the N-terminal site region, four competent sites for glycosylation, and five cleavage sites that might be cut by kex2 protease. The mature KHR toxin does not have clearly hydrophobic regions, and so its mode of action is probably different from that of K1 (142).

KHS encodes for a 79-kDa precursor with a hydrophobic N-terminal sequence that is probably spliced to produce the mature toxin. The mature KHS toxin shows three clusters of hydrophobic amino acid sequences that might have an ionophore function similar to that of K1 or K2 toxins. These killer genes might also contain regions responsible for immunity of the yeast to its own killer toxin (144).

Ustilago maydis Killer System

dsRNA viruses with segmented genomes are responsible for the killer phenotype in the maize smut fungus *Ustilago maydis* (204). Killer strains 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 (203). 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) killer strains have been described (35, 205). The dsRNAs are encapsidated in 43-nm capsids that have a major capsid peptide of 73 to 75 kDa (Fig. 2).

The H dsRNAs are packaged singly and encode capsid proteins that are similar if not identical. These proteins are encoded by the H2 segment (4.6 kb) in P1 and P4 and by the H1 segment (6.2 kb) in P6. They apparently lack any relatedness (283). Other H fragments possibly encode capsid-related polypeptides of 100 to 128 kDa, whose production in vivo would require posttranslational cleavage to yield the 75-kDa capsid protein (331). Besides coat formation, the essential functions for replication of all segments and the maintenance of the M and L segments seem to be associated with the H dsRNAs (206, 207).

The M dsRNAs may be encapsidated singly or in combinations of multiple segments and are thought to code for killer toxin production (35). On the basis of genetic and in vitro translation experiments, one of the M segments in each killer strain appears to code for the killer toxin: M2 in P6, M1 and/or M2 in P1, and M2 in P4 (206, 207, 283, 331, 388). P1M1, P4M2, and P6M2 dsRNAs show a high degree of sequence homology in a noncoding region of unknown function near the 3′ terminus of the positive strand (105, 270, 388). KP toxins have different specificities, so that cells resistant to one can be susceptible to another. Like the K toxins of *S. cerevisiae*, they seem to be synthesized as preprotoxins that are successively processed intracellularly to yield the mature secreted bipartite (KP1 and KP6) and single subunit (KP4) toxins. Of these toxins, KP6 is the best characterized, since it has been highly purified. Its cDNA has been cloned, sequenced, and expressed in both homologous and heterologous systems (112, 273, 388, 389). This toxin consists of two nonglycosylated polypeptides: α (78 amino acids; 8.6 kDa) and β (81 amino acids; 9.1 kDa) (273, 388). They are cleaved by kex2-like and protease-processing events from a preprotoxin of 219 amino acids (24.1 kDa), encoded on the M2 dsRNA and comprising a signal sequence, the α polypeptide in a glycosylated form, an interregion, and the β polypeptide (388). In the mature toxin, α and β are not covalently linked. They interact as monomers with

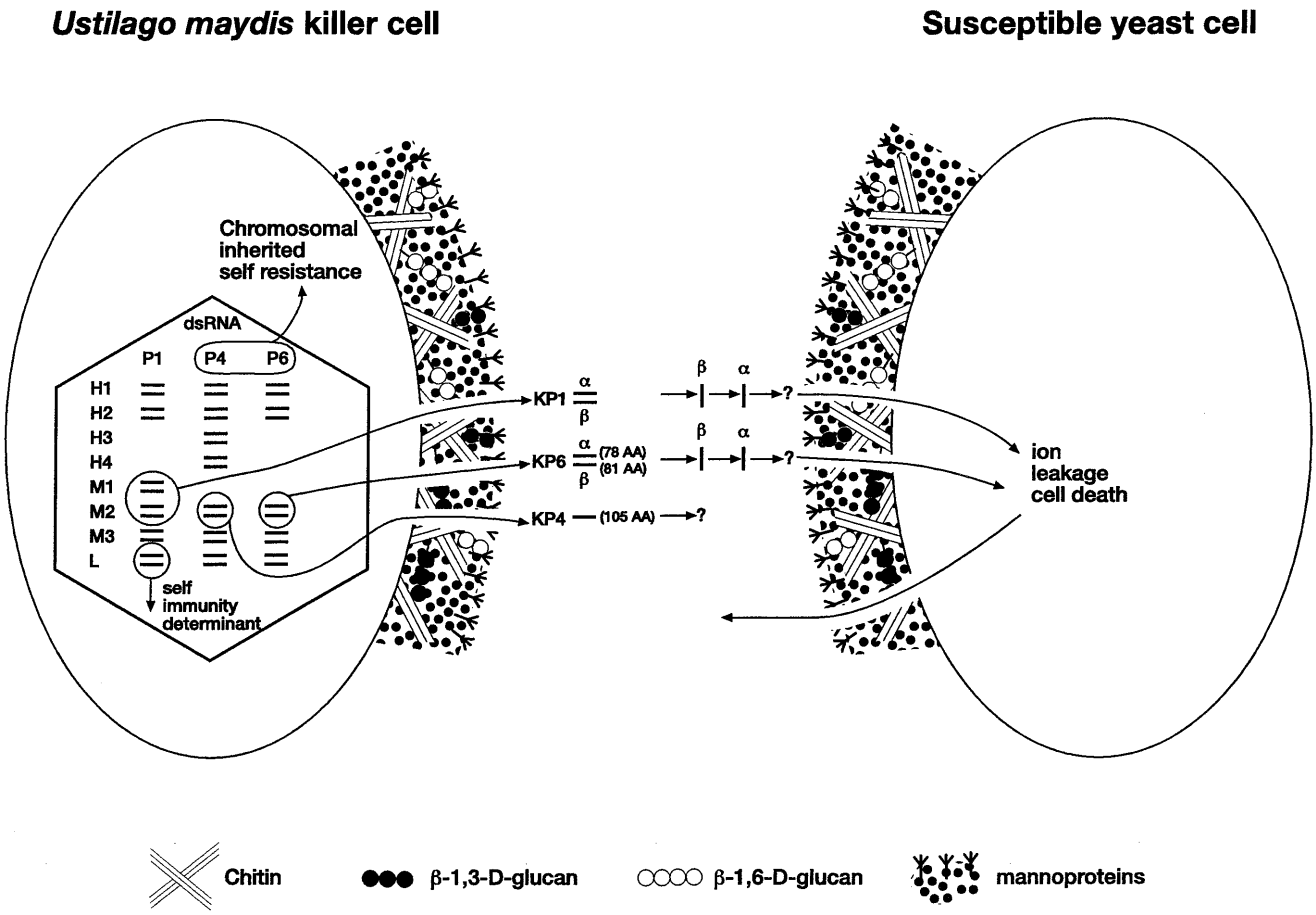

FIG. 2. Sketch of the genetics, toxigenesis, immunity, and activity of the *U. maydis* killer system. AA, amino acids.

the susceptible cells independently and in a sequential manner, with the α subunit initiating the interaction and the β subunit exerting its effect only on cells previously exposed to α. Receptors on the cell wall have not yet been identified, but the toxin binds to the cell wall. The spheroplasts are not susceptible to the toxin (368). The mode of action is unknown, but the α subunit contains three hydrophobic regions that are believed to be structurally and functionally important. This subunit shows some sequence similarity to scorpion neurotoxins and cytotoxins. It contains eight cysteines and requires intramolecular disulfide bridges for activity, which may lead to the formation of ion channels. The β subunit has a hydrophobicity profile very similar to that of K1 (388).

Like KP6, the KP1 toxin is bipartite and the α and β subunits are not covalently linked but act independently as monomers. The hydrophobicity profile of the β subunit is almost identical to that of K2 and the subunit appears to be glycosylated (388).

The KP4 toxin has been recently purified and characterized (127, 128). The cDNA derived from M2 dsRNA has been cloned, sequenced, and expressed in *S. cerevisiae* (270). This toxin differs from the other known killer toxins in several respects apart from its minimal primary sequence similarity. It is synthesized as a preprotoxin of 127 amino acids (13.6 kDa) without any sites for N-linked glycosylation. It is not processed by any of the known secretory proteases, other than the signal peptidase. The preprotoxin has a highly hydrophobic N terminus with a probable signal peptidase cleavage site after Ser^{22}. The mature toxin is secreted as a single polypeptide of 105 amino acids (11.1 kDa) with a pI of 8.1 and with 10 cysteines possibly involved in disulfide binding that is probably important in the activity of the toxin. This toxin, despite previous reports, does not appear to be glycosylated (127, 128, 270). Its mode of action is still unknown.

U. maydis killer strains are not susceptible to their own toxins, but, unlike in *S. cerevisiae*, the immunity appears unrelated to the precursors or metabolites of the toxins. In P1 strains, the immunity to the toxin is associated with the L segment, while all known P4 and P6 strains are resistant to the respective toxin by virtue of recessive chromosomal mutations (112, 207, 270, 272). No single-gene mutation rendering *Ustilago* killer strains resistant to more than one toxin is known (204). This situation is unlike that for some of the previously cited *kre* mutations with respect to K1 and K2 toxins in *S. cerevisiae*. Little is known about the L segments except that they are almost identical to the terminal one-third of the respective larger M segments and are probably derived from a posttranscriptional cleavage of M positive-strand RNAs (65, 105).

Kluyveromyces lactis Killer System

The killer phenomenon in the petite negative yeast *Kluyveromyces lactis* was discovered by Gunge et al. during screening for new plasmids from various yeast species (148). This finding was unexpected because the phenomenon was associated with DNA linear plasmids. Killer strains always contain 50 to 100

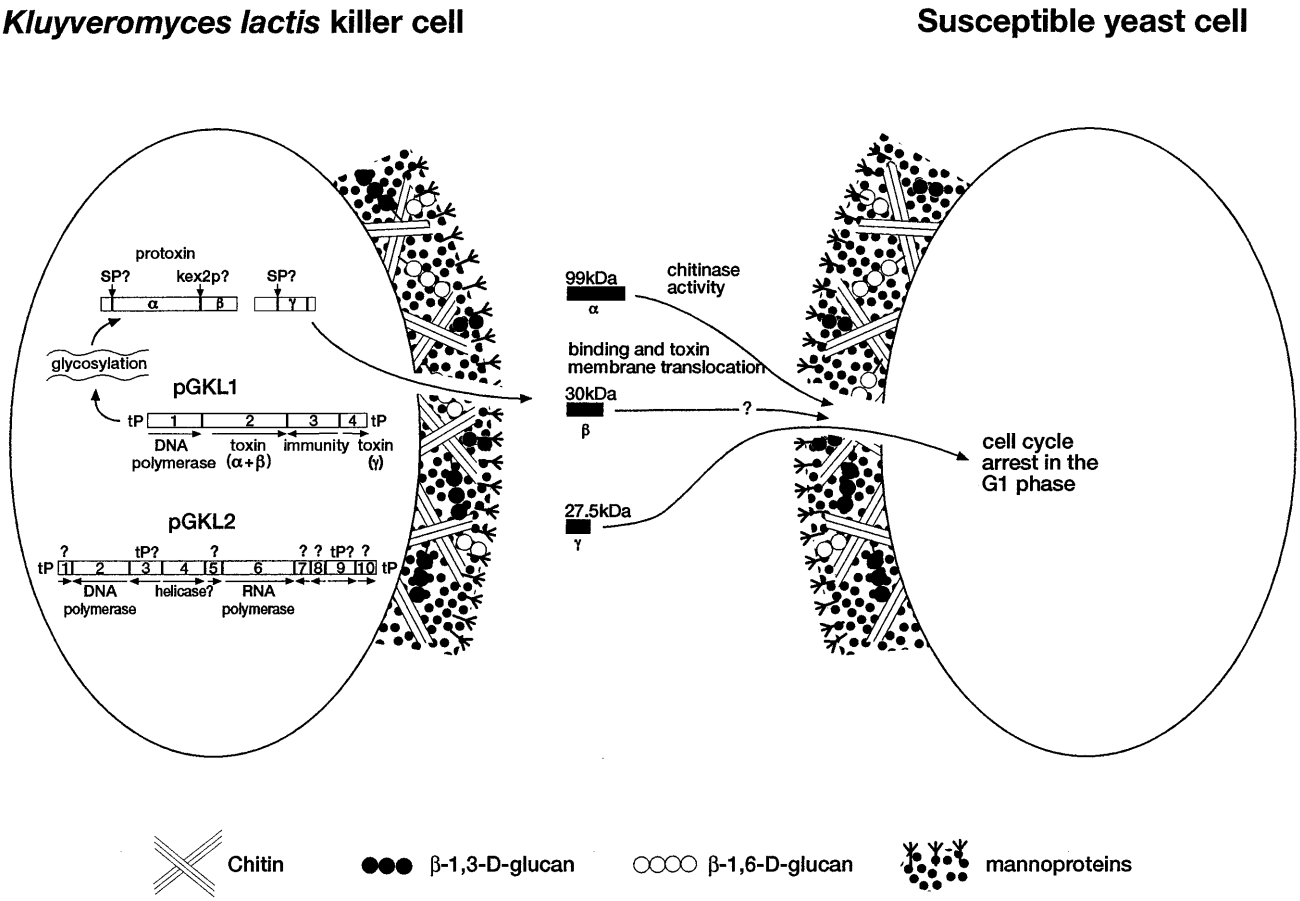

FIG. 3. Sketch of the genetics, toxigenesis, immunity, and activity of the *K. lactis* killer system. pGKL1 and pGKL2 refer to cytoplasmically inherited linear plasmids.

copies per cell of each of two cytoplasmically inherited linear plasmids designated pGKL1 (k_1) and pGKL2 (k_2), which are 8,874 and 13,447 bp in size, respectively (Fig. 3). Both plasmids have been totally sequenced, and their organization, replication, and expression have been recently reviewed (124, 149, 170, 340, 360, 362, 363, 417). Four ORFs, whose functions are mainly understood, are present in the smaller plasmid. Two (ORF2, 3,440 6p; ORF4, 749 bp) encode the precursors of the subunits of the killer toxin, a third (ORF3; 1,286 bp) is involved in the immunity phenotype in a still unknown manner, and the fourth (ORF1; 2,987 bp) codes for a DNA polymerase (123, 188, 361, 398). ORF3 is encoded on the strand opposite that encoding the other ORFs, and the plasmid is organized in a highly compact manner (358). The ORFs form up to 95% of the DNA. In contrast, no definite functions have been assigned to any of the 10 ORFs of the larger plasmid, which is indispensable for k_1 and is involved in the control of replication and maintenance of both plasmids. These ORFs comprise 97% of the genome, with frames overlapping extensively on both strands (400). ORF2 of k_2 shares homology with ORF1 of k_1 and is presumed to encode a DNA polymerase involved in the replication of k_2. ORF6 codes for an RNA polymerase, and ORF4 codes for a probable DNA helicase, while ORF5 codes for a small neutral protein of 18 kDa of as yet unidentified function but essential for plasmid integrity and/or maintenance (330, 363, 440). ORF1 has no function in the expression of immunity, as had been supposed (363). It is not needed for killer plasmid replication and maintenance but probably is required for the maintenance of normal copy numbers (329). The 14-kDa histone-like protein, encoded by ORF10, can bind to both k_1 and k_2 plasmids, presumably acting as a structure stabilizer rather than as a regulatory protein. As some linear DNA viruses, both plasmids have specialized structures at their ends that are involved in the mechanism of replication (245, 311, 452). There are terminal inverted repeats of 202 bp in k_1 and 184 bp in k_2, whose 5′ ends are covalently associated with 28- and 36-kDa terminal proteins, respectively (170, 197, 358). Of the k_2 plasmid ORFs with no assigned functions, ORF3 and ORF9 are sufficiently large to encode either the k_1 or k_2 terminal protein (417). As mentioned above, k_1 cannot exist without k_2, requiring the RNA polymerase encoded by k_2, which is necessary for the transcription of both plasmids. DNA replication probably depends on protein priming and a strand displacement mechanism similar to that of the adenoviruses (116). Following the attachment of a terminal protein linked to a nucleotide that serves as the primer, the specific DNA polymerase replicates the respective plasmid, proceeding unidirectionally in the 5′-to-3′ direction and displacing one of the parental strands. Pairing of the inverted repeats in this strand creates a partial double strand where the second-strand displacement and replication start. The cytoplasmic localization of the plasmids implicates the existence of an independent expression system, in which the RNA polymerase encoded by ORF6 of k_2 must play a central role, recognizing as specific

cytoplasmic promoters some of the upstream conserved sequences in the 5′ noncoding regions of all ORFs (124, 318, 359, 440).

Nothing is known about the mechanism of segregation of the plasmids to daughter cells at division. No chromosomal genes, analogous to the *MAK* genes in *S. cerevisiae*, have been identified. The high copy number of the plasmids probably allows their random distribution to the daughter cells, and restoration of the number could be ensured by plasmid replication independent of nuclear replication.

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 nonkiller strains of *K. lactis* (148).

The toxin consists of three subunits: a polypeptide with a single asparagine-linked oligosaccharide unit, designated α (99 kDa), and two smaller unglycosylated components, β (30 kDa) and γ (27.5 kDa), encoded by k_1 (361). ORF2 encodes a precursor (128 kDa) of the α and β subunits, comprising an N-terminal signal peptide and consensus cleavage sites for signal peptidase or a kex2-like protease. The precursor is targeted to the endoplasmic reticulum, where it is glycosylated, transported to the Golgi apparatus, and processed by a kex2-like protease to form mature subunits. At the same time, the N-terminal signal peptide is cleaved at the carboxyl side of Arg^{29}. ORF4 encodes the precursor of the γ subunit containing an N-terminal signal peptide cleaved from a peptidase during processing (71, 147, 361, 399, 427). The mature toxin leads to the permanent arrest of susceptible cells in the unbudded (G_1) phase of the cell cycle, in such a manner that they can never resume mitotic division (147, 428). 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 (378, 428). The nonviable toxin-treated cells maintain a membrane potential, in contrast to the effect of other killer toxins that act as ionophores. The mode of action of the toxin is still poorly understood. However, two effects, arrest in G_1 and loss of viability, are apparently mediated by different mechanisms. Only the γ subunit appears to be required to arrest proliferation, acting against an unidentified intracellular target (53). The α subunit shows sequence homology to both plant and bacterial chitinases and has the essential chitinase activity that is required for the toxin to act on susceptible yeast cells (52, 425). As this activity cannot mediate the cell cycle arrest induced by the toxin, it is most probably required by the γ subunit to gain entry to the susceptible cell. It is probably involved in the interaction with a carbohydrate receptor in the cell wall or, alternatively, in the degradation of cell wall carbohydrate structures, thus providing the toxin with a route of entry into the cell.

More recent data seem to indicate that mutants defective in chitin synthetase III are resistant to the toxin (45). Chitin may represent the cell wall receptor for the killer toxin, at least in *S. cerevisiae* (387). After the toxin binds to the chitin in the cell wall, it is likely that part or all of the molecule is translocated across the membrane into the cytoplasm. The function of the β subunit is still unknown. However, since its molecule shows four hydrophobic regions, it has been hypothesized that this subunit may play a role, together with α, in the binding and membrane translocation of the toxin, allowing the γ subunit to enter the cell or resulting in the loss of viability of the treated cells, as the γ subunit alone can account only for the G_1 arrest. The characterization of toxin-resistant mutant strains will allow a better understanding of the molecular basis of the action of the toxin (53, 387). Two genes, encoding a tRNA-(Glu3) and a novel as yet unidentified polypeptide, seem to control the susceptibility to G_1 arrest induced by the *K. lactis* toxin in *S. cerevisiae*. The polypeptide probably is involved in the synthesis of the target of the toxin (54).

Pichia and *Williopsis* Killer Systems

Killer strains with a wide spectrum of intergeneric killing have been found in spore-forming ascomycetous yeast species of the genus *Pichia* and the genus *Hansenula* (now *Williopsis*) (212, 296, 448, 453). These strains display a wide range of activity against *S. cerevisiae* and other yeasts, as well as against the dimorphic yeast *C. albicans*. It is possible that multiple toxins, active against different susceptible strains, are produced by a single killer strain (420). The killer phenotype in these yeasts is heterogeneous and was thought to be chromosomally inherited until linear dsDNA plasmids, similar to those in *K. lactis*, were discovered in *Pichia inositovora* and *P. acaciae* (162, 224, 442).

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* (224). Only two of them (p*Pin*1-1 and p*Pin*1-3) seem to be associated with the killer phenotype, while the loss of p*Pin*1-2 has no effect on toxin production or susceptibility. Cured isolates lose the ability to produce toxin but do not become detectably susceptible to toxin, so the immunity function does not appear to be associated with the plasmids but is probably chromosomally encoded. The killer toxin apparently is an acidic heat-labile glycoprotein whose characterization and range of actions have not yet been determined (162).

P. acaciae killer strains have been shown to possess two linear plasmids, designated p*Pac*1-1 (13.6 kbp) and p*Pac*1-2 (6.8 kbp). These plasmids are quite similar in both function and structural organization to those found in *K. lactis* (26, 442). p*Pac*1-2 was found to hybridize to k_1 ORF2 and is thought to be involved in toxin production (26). 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 G_1 cell cycle arrest, acting at a pH optimum between 7.0 and 7.5 (241). This toxin shows a wide range of activity, differing from but overlapping with that of *K. lactis*. Cured *K. lactis* strains that lose k_1 become susceptible to both toxins, while cured *P. acaciae* strains lose immunity to their own toxin only, suggesting a different mechanism of immunity in the two yeasts. 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 extrachromosomal elements (26).

Other killer phenotypes are associated with chromosomal genes. The *P. kluyveri* killer toxin, a 19-kDa acidic glycoprotein, induces the formation of ion-permeable 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. A novel type of killer toxin produced by the halotolerant yeast *P. farinosa* has been recently described (189, 248, 249, 381). 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* preprotoxin resembles the *S. cerevisiae* K1 toxin

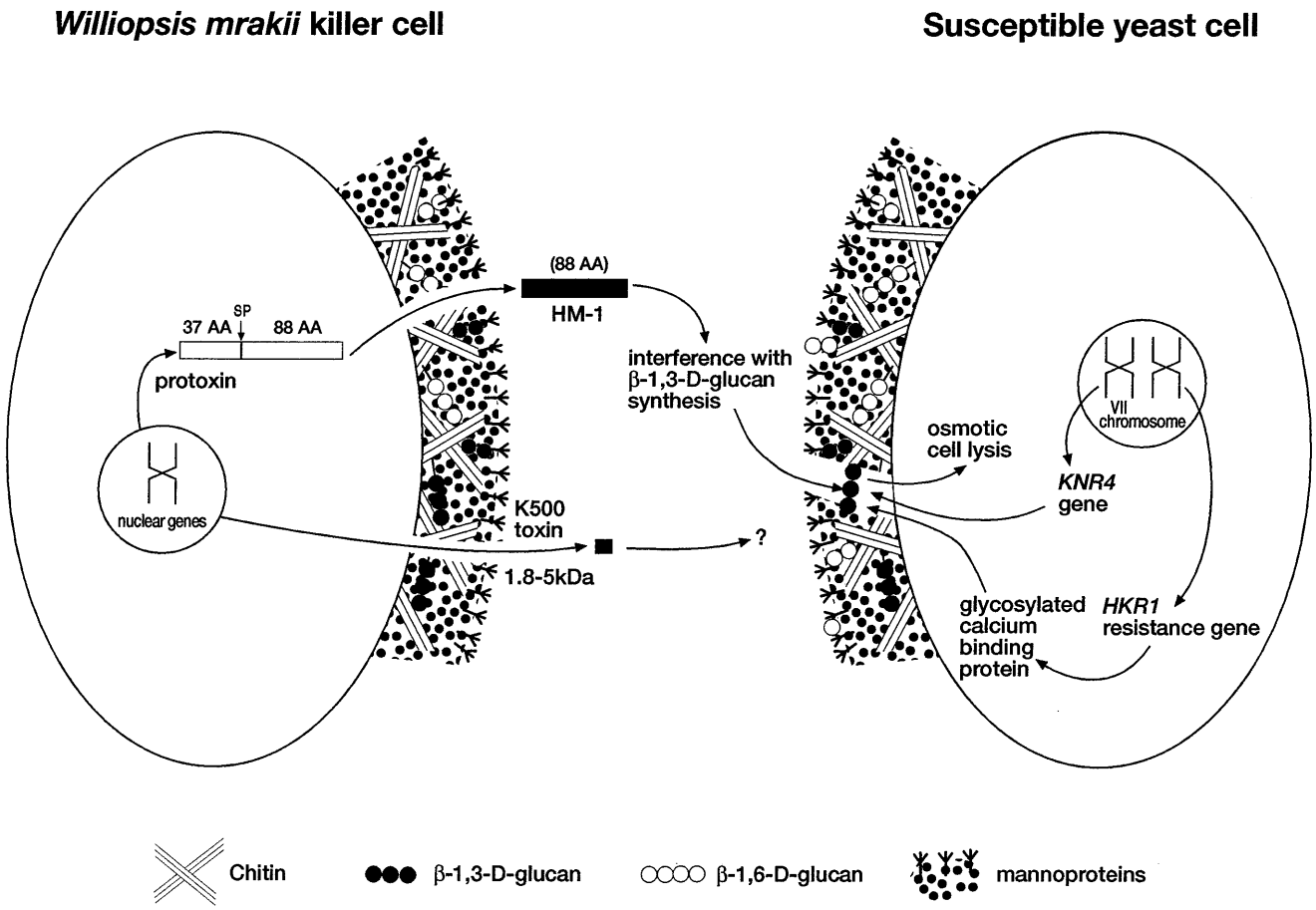

FIG. 4. Sketch of the genetics, toxigenesis, and activity of the *W. mrakii* killer system. AA, amino acids.

in overall structure, hydrophobicity profile, and processing, suggesting that the target of the toxin is the membrane. However, preliminary experiments on *kre* mutants suggest that molecules other than β-1,6-D-glucan could act as toxin receptors on susceptible cells.

The *Pichia* and *Williopsis* killer systems are of great interest because, unlike the *S. cerevisiae* K1 toxin, which targets the β-1,6-D-glucan, some of the killer toxins that they produce interfere with the synthesis of β-1,3-D-glucan, the major cell wall polysaccharide polymer involved in determining cell morphology and in maintaining osmotic integrity (Fig. 4).

At least two different killer toxins have been described in *Williopsis mrakii*: a toxin designated HM-1 or HMK, first described in 1983; and a second one, described more recently and designated K-500 (8, 171). Unlike most of the reported toxins, HM-1 is a basic unglycosylated polypeptide (pI 9.1); is composed of 88 amino acids (10.721 kDa), 10 of which are cysteines; and shows high thermostability (100°C for 10 min) and pH stability between pH 2 and 11 (446). The exceptional stability of this cysteine-rich molecule appears to be due to the existence of many disulfide bonds, which also seem to be essential for its biological activity, as demonstrated by the inactivation of the toxin by reducing agents such as 2-mercaptoethanol and dithiothreitol. HM-1 kills susceptible strains by a unique mechanism, presumably involving interference with the synthesis of β-1,3-glucan, thus rendering the wall osmotically fragile or defective and ultimately resulting in lytic cell death (447). The involvement of cell wall β-glucans in the initial step of the toxin action is strongly suggested by other observations. HM-1 kills intact cells but not protoplasts; cells that lack the functional KRE6 allele, which have reduced levels of β-1,6-glucan, are resistant to higher concentrations of the toxin (193). The cytocidal action of HM-1 is blocked by either purified β-1,3- or β-1,6-D-glucan, suggesting that this toxin–β-glucan interaction is required for the toxin activity. HM-1 inhibits the in vitro activity of β-1,3-D-glucan synthase, suggesting that the toxin can perturb the synthesis of the yeast cell wall by inhibiting the glucan synthesis at the budding sites or the conjugating tubes, which results in cell lysis (383). The overexpression in *S. cerevisiae* of an essential gene, designated *HKR1* (*Hansenula* killer toxin-resistant gene 1), allows it to overcome the effect of the toxin by endowing the susceptible cells with resistance. *HKR1* encodes a high-molecular-weight calcium-binding glycosylated type I membrane protein that regulates both cell wall β-glucan synthesis and budding pattern (443). This suggests that changes in the intracellular calcium concentration may be associated with the toxin effect (194).

W. mrakii killer toxin was used to select resistant *S. cerevisiae* mutants showing structural defects in the cell wall. A nonessential gene, designated *KNR4*, which is involved in the β-1,3-D-glucan synthesis, was cloned and characterized from one of these mutants. This gene, mapping to the right arm of chromosome VII, probably encodes an auxiliary protein involved in the coordinated regulation of β-1,3-D-glucan and chitin syntesis. This protein can act either directly on other cell wall genes or indirectly through another cell wall regulator. Disruption of

the *KNR4* gene results in reduced levels of both β-1,3-D-glucan synthase activity and glucan content in the cell wall, in increased cellular resistance to β-1,3-D-glucanase digestion and to the toxin, and in greater cellular susceptibility to osmotic destabilization (173).

The *HMK* gene, encoding HM-1 toxin, and the *HSK* gene, encoding a very similar killer toxin in *W. saturnus*, have been cloned and sequenced (198, 267). They encode precursors of 125 and 124 amino acids, respectively, showing an N-terminal signal sequence of 37 amino acids, which may be removed by a signal peptidase, followed by mature proteins of 88 and 87 amino acids, respectively. These may be cleaved off by a kex2p-like protease. The preprotoxins do not confer immunity, and the mature toxins show an extensive sequence homology. The molecular mechanisms of killing are probably identical, but the different primary structures could be responsible for the difference in their killing spectra. The unusual stability of HM-1 has made it possible to assay its antimicrobial activity against a wide range of yeasts and filamentous fungi, whose different cell wall compositions could explain the differences in susceptibility to the toxin (449).

In contrast, the K-500 killer toxin is an acidic polypeptide with a relative molecular mass between 1.8 and 5.0 kDa. It is readily inactivated by high temperature and pH values above 4.0. It possesses extensive anti-*Candida* activity. The toxin has not yet been completely characterized, and its killing mechanism remains unclear. It is significantly smaller than any of the other known yeast killer toxin polypeptides. The observed dose-response of susceptible cells to the effects of the toxin supports the hypothesis of an interaction with cell wall receptors. There is some evidence that the toxin can act in a manner similar to *S. cerevisiae* K1 and *P. kluyveri* toxins by producing channels in the susceptible cell and thus promoting the loss of ions that leads to cell lysis.

The killer system that has attracted the greatest attention is the one in *P. anomala*. This attention results from its 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 (288, 292). Little is known about the nature and mode of action of the *P. anomala* killer toxin(s). Two killer strains active against *Zygosaccharomyces rouxii*, whose toxins have been shown to be high-molecular-mass (300-kDa) glycoproteins active under conditions of high NaCl concentration and chromosomally inherited, have been reported (190).

A killer toxin, purified from a strain (WC 65) of *P. anomala*, has been characterized and demonstrated to be an acidic glycoprotein of 83.3 kDa, stable between pH 2.0 and 5.0 (326). The toxin shows saturation kinetics at increased toxin concentrations, suggesting a receptor-mediated action (325). 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. One of them (probably a surface β-1,6-D-glucan-related binding site) binds noncompetitively, and the other binds competitively. The role of a cell wall receptor is also supported by the decreased susceptibility to the toxin of glucan-deficient mutants and by the specific fluorescence of the walls of anti-toxin antibody-treated susceptible *C. albicans* cells (327). A second toxin produced by the killer strain *P. anomala* ATCC 96603, previously known as UP 25F, has been extensively investigated for its antimicrobial activity. This toxin is supposed to be a glycoprotein encoded by nuclear genes. It acts on a wide spectrum of susceptible microorganisms that are characterized by the presence of specific cell wall receptors and lack of immunity systems (288, 295). The toxin cell wall receptors in *C. albicans* seem to be distributed mainly in budding cells and germination tubes, supporting previous findings that the *P. anomala* killer toxin is more active against young yeast cells. Killer cells are immune to their own toxin while possessing specific cell wall receptors, but the mechanisms of killing and immunity still remain unknown. A fully active toxin is secreted in the presence of tunicamycin, suggesting that N glycosylation is not involved in either toxin secretion or killer activity (56). Ultrastructural studies on the secretion of the toxin, carried out by immunoelectron microscopy with a specific monoclonal antibody, suggest that it undergoes heterogeneous secretion by the killer cells and concentration in the cell wall layers before being exported outside the cells (55, 57). The most important characteristic of this toxin is its wide range of activity, which suggests the existence of a ubiquitous form of yeast toxin receptor, probably constituted by β-glucan. The occurrence of specific killer toxin receptors at the surface of *Pneumocystis carinii* was hypothesized by considering the probable presence of β-1,3-glucans in its cell wall (81, 238). This widespread eukaryotic microorganism is found in the lungs of humans and mammals and becomes an important opportunistic agent of severe pneumonitis in immunocompromised patients, especially those with AIDS. *Pneumocystis carinii*, previously classified among the protozoans, is now considered to be a fungus on the basis of rRNA sequence homology (92, 282, 371, 372, 419). Its susceptibility to the *P. anomala* killer toxin has been demonstrated. The killer toxin induces a marked specific inhibition of the in vitro attachment of rat- or mouse-derived *Pneumocystis carinii* to Vero cells, presumably due to the occurrence of specific receptors on the cell wall of the microorganism (5). These receptors were visualized by immunofluorescence with a specific anti-toxin monoclonal antibody on *Pneumocystis carinii* organisms treated with killer toxin (Fig. 5). An inhibitory effect of the toxin has also been demonstrated on *Pneumocystis carinii* infectivity to SCID mice (345, 346). These effects could be due either to the death of the toxin-treated parasites or to a specific inhibitory effect of the toxin on the adhesion mechanisms of *Pneumocystis carinii* at the surface of

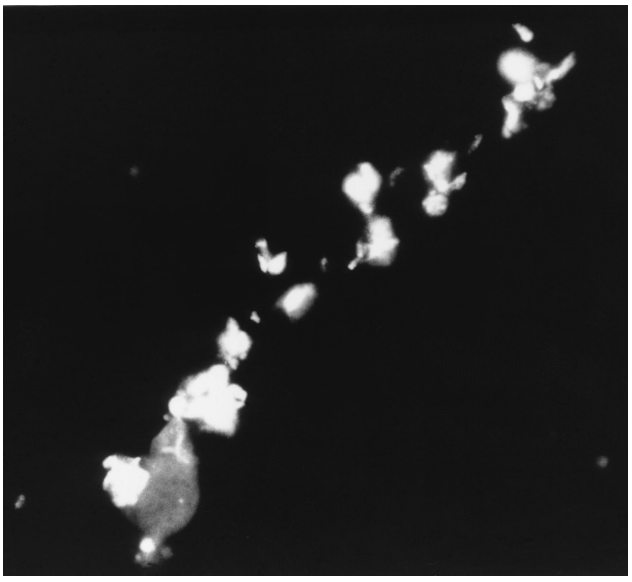

FIG. 5. Putative cell wall receptors in *Pneumocystis carinii* visualized by immunofluorescence with a *P. anomala* killer toxin and a monoclonal antibody against the toxin.

host cells. A real killer effect of the *P. anomala* killer toxin against *Pneumocystis carinii*, however, was demonstrated by experiments with radiolabelled para-aminobenzoic acid. The inhibition of the uptake of the folate precursor proved that there was a marked loss of viability of the microorganisms treated with the killer toxin (179). Because to date there have been no reports of killer toxin activity against protozoans, the susceptibility of *Pneumocystis carinii* to the *P. anomala* toxin might further confirm the fungal nature of this parasite.

Other Yeast Killer Systems

As outlined above, yeast killer strains have been successively identified in most of the yeast genera and the killer phenotype has been associated with the genetic determinants described above. dsRNA mycoviruses, similar to those in *S. cerevisiae*, have been detected in the yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii*. An M-dsRNA satellite appears to contain the genetic information for toxin production. However, expression of immunity, at least in *Z. bailii*, may be dependent on an additional Z-dsRNA, resembling a new replicon independent of an L-A helper virus (307, 308, 337, 461). Four dsRNA molecules associated with virus-like particles, encoding a killer system, have been isolated from *Phaffia rhodozyma* (64).

Recently, *Cryptococcus humicola* strains secreting small, acidic, thermostable, methanol-soluble, chromosomally encoded toxins, specified as microcins, have been isolated, suggesting that the killer phenomenon may be due to the excretion of two types of killer toxins: mycocins and microcins (139). In addition, chromosomally encoded killer systems have been identified in almost all of the genera of yeasts comprising opportunistic pathogens, such as species of *Candida*, *Cryptococcus*, and *Torulopsis*, even though it is unlikely that killer toxins are important as virulence factors (191, 208, 418, 432, 450, 451).

A novel killer toxin from a *Candida* wild strain proved to be formed from two components, I and II, represented by glycoproteins of 36 kDa each. Toxin II proved to be less active than toxin I, which showed strong killer activity against *C. glabrata*, *P. anomala*, *K. lactis*, *P. membranaefaciens*, *Rhodotorula rubra*, and *S. cerevisiae*. Bacteria and fungi, as well as other yeasts, were not affected by toxin I (450, 451).

The principal characteristics of the genetics and the structure of killer toxins, their specific receptors, and the recognized mechanisms of action of the most investigated yeast killer systems are reported in Table 1.

ECOLOGY OF YEAST KILLER SYSTEMS

Environmental Yeast Killer Systems

The nature of the yeast killer phenomenon implies a potential role for competition, considering that yeast killer toxins may prevent antagonistic microorganisms from gaining access to resources that would provide a selective advantage during the early phases of microbial growth (76, 96, 137). Yeast killer toxins are produced optimally by growing cells and are exquisitely active against cells in the same stage when nutrients are available and pH is low.

The ecological role of the yeast killer phenomenon (toxin production and susceptibility) has been extensively studied in yeast communities, particularly in decaying stems and fruits and in slime growth on trees (366). In particular, fruits appear to be very important habitats for the killer phenomenon within yeast communities, since one-quarter of the yeast strains isolated from them are killers. This habitat is characterized by a low pH and high sugar concentration. Fruits may be considered relatively accessible environmental sites visited by a variety of potential yeast vectors such as bees, beetles, birds, and other animals. In nature, some killer yeasts require efficient vectors such as insects, and *Drosophila* spp. in particular, to transfer them from one rot to another (129, 136). Coadaptation and coevolution imply nutritional benefits for the insects that feed on fruits and the advantages of transfer for the yeasts (14, 365). Adult drosophilids, feeding on fruits in nature, usually carry yeasts and contaminate artificial media when deposited on them (129). The role of the killer phenomenon among yeasts in natural habitats appears to be of special relevance in the open system of fruit communities, in which various insects may carry different colonizing yeasts. In contrast, the role of killer systems seems minimal in maintaining yeast distinction in sympatric habitats. Significantly, resistant rather than susceptible yeast strains are usually found in fruits (366).

It is intriguing that some dominant yeasts isolated from fruit lose their killing properties after a short time in culture in a laboratory, probably owing to the loss of plasmids as a result of elevated incubation temperatures. This observation should alert investigators about misinterpretating the killer-susceptible interactions observed in yeasts obtained from culture collections.

Studies based on killer-susceptible interactions showed that under proper conditions of evaluation, the killer phenomenon occurred more frequently among yeasts from different localities than from within the same habitat. A possible interpretation of this phenomenon is that a certain level of adaptation to killer resistance would be expected under limited selective pressure at one place and time. Generally, natural communities of yeasts are made up of only one predominant killer species, even though susceptible strains are more widespread than the killer counterpart. Few species, if any, appear to be immune to all yeast killer toxins. Ecologically defined natural yeast killer systems showed a definite seasonal fluctuation in their occurrence that markedly increased during the cooler winter period, possibly because of the seasonal nature of fruit production at certain latitudes (366).

Studies of some environmental factors, such as the pH of the habitat in which the killer yeast lives, have shown that killer and susceptible yeasts are not independently distributed and that some killer toxins function in nature to limit the proliferation of other yeasts in the same community. The killer factor has been demonstrated to be a competitive mechanism of interference in yeasts associated with cacti. The species composition of yeast communities is distinct in cacti and surrounding habitats and varies with the type of plants (129, 364). Ecology has proved to be a more important factor than phylogeny in determining the killer phenotype of yeast communities. Because of the discrete nature of the cactus habitat and the limited number of interacting yeast species, cacti are fruitful models for studying interactions in communities of killer and susceptible yeasts, even though not all of the processes that influence yeast community dynamics are fully understood (host cactus chemistry, yeast-vector mutualism, and potential synergism) (365). The possibility exists that yeast interference-competition and mutualism are both important determinants of the composition of yeast communities and are related to variations among the habitats in which the yeasts coexist.

Further evidence of the role that killer yeasts play in nature in excluding competitor yeasts from particular habitats should come from field tests. Experimental studies of yeast interference competition in cacti have demonstrated that the composition of the yeast community differed in the presence of a

TABLE 1. Principal characteristics of the most extensively investigated yeast killer systems

Genetic basis	Killer system	Genome	Genome size (Kbp)	Killer toxin	Killer toxin size (kDa)	Receptor	Mechanism of action
dsRNAs encapsidated in virus-like particles	*Saccharomyces cerevisiae*						
	K1	M1	1.8	αβ dimer	19.0	β-1,6-D-Glucan	Increase of membrane permeability to ions
	K2	M2	1.5	αβ dimer	21.5	β-1,6-D-Glucan	Increase of membrane permeability to ions
	K28	M28	1.9	αβ dimer	21.5	α-1,3-Mannose	Inhibition of DNA synthesis
	Ustilago maydis						
	P1	M1/M2	1.4	αβ dimer	19.0	—	Increase of membrane permeability to ions
	P4	M2	0.98	Monomer	11.1	—	Increase of membrane permeability to ions
	P6	M2	1.2	αβ dimer	17.7	—	Increase of membrane permeability to ions
	Hanseniaspora uvarum	M	—[a]	—	—	—	—
	Zygosaccharomyces bailii	M	—	—	—	—	—
	Phaffia rhodozyma	M	—	—	—	—	—
Linear dsDNA plasmids	*Kluyveromyces lactis*	pGKL1,L2	8.8–13.4	αβγ trimer	156.5	Chitin	Cell cycle arrest in G_1, chitinase activity
	Pichia inositovora	p*Pin*1-1,1-3	18–10	—	>100	—	—
	Pichia acaciae	p*Pac*1-1,1-2	13.6–6.8	Trimer	~190	Chitin	Cell cycle arrest in G_1, chitinase activity
Nuclear genes	*Saccharomyces cerevisiae*						
	KHR	*KHR*	0.9	Monomer	20	—	Increase of membrane permeability to ions
	KHS	*KHS*	2.1	Monomer	75	—	Increase of membrane permeability to ions
	Pichia kluyveri	—	—	Monomer	19	—	Increase of membrane permeability to ions
	Pichia farinosa KK1	*SMK1*	0.6	αβ dimer	14.2	—	Increase of membrane permeability to ions
	Williopsis mrakii						
	HM-1	*HMK*	0.85	Monomer	10.7	Cell wall β-glucan	Inhibition of β-1,3-glucan synthesis
	K-500	—	—	Monomer	1.8–5.0	Cell wall β-glucan	Increase of membrane permeability to ions
	Pichia anomala WC 65	—	—	Monomer	83.3	Cell wall β-glucan	—

[a] —, not known.

killer or a nonkiller strain. The killer yeast was present in a higher density than the nonkiller strain because of selection against susceptible yeasts. The density of the population of susceptible yeasts grown in the presence of the killer strain was much lower than that of the same susceptible yeast strain grown in association with the nonkiller strain (130). The probability that a killer toxin produced by a yeast may kill a certain susceptible yeast would also depend on ecological characteristics such as the region, the host plant, and the habitat from which both the killer and susceptible yeasts were collected.

A very high percentage of strains of *P. kluyveri* isolated from all parts of the United States showed killing activity against natural susceptible isolates or those from culture collection. The killer activity appeared to be evenly distributed across distinct locales, regions, and the continent (367). Killer strains of *S. cerevisiae* and *P. anomala* markedly inhibited the growth of certain wood decay basidiomycetes and plant-pathogenic fungi, suggesting that such yeasts might have a potential as novel antimycotic biocontrol agents (420). It has been suggested that the genetic information for toxin production could be integrated into and expressed from the genome of maize plants (*Zea mays*) to render such transgenic plants resistant to the attack of yeast phytopathogens such as *U. maydis* (203). One toxin encoded by *U. maydis*, the KP6 killer toxin, was shown to be synthesized by transgenic tobacco plants containing the viral toxin cDNA under the control of a cauliflower mosaic virus promoter. The two components of the KP6 toxin (α and β) proved to have identical activity to those found in the toxin secreted by *U. maydis* cells. They were isolated from the intracellular fluid of the transgenic tobacco plants. The systemic production of viral killer toxin in crop plants may provide evidence to support a new approach to engineered biological control of fungal pathogens in plants (199).

Suboptimal conditions for screening may result in an under-

estimation of the number of killer and susceptible strains in nature. Natural killing activity does not always occur at the usual pH values at which the screening is performed, and additional killer phenotypes may have escaped attention because the proper susceptible strain was not used. Moreover, yeasts with strong killer activity are normally reputed to display a wider spectrum of antifungal activity than are yeasts with weak killer activity. Thus, the real number of yeast strains considered susceptible might depend on the level of yeast killing activity (374).

Industrial Yeast Killer Systems

In recent years, considerable efforts have been made to introduce new technologies dealing with the adoption of biological processes into the food industry. Optimization of fermentation procedures may result in food, wine, grape juices, and beer of improved quality (412). Particular attention has been paid to the use of killer yeasts as selected starters in fermentation processes. Some strains already have been applied to production, whereas others are still in an experimental stage. Alcoholic fermentation, causing the transformation of sugars to ethanol and CO_2, and malolactic fermentation, leading to lactic acid and CO_2 production, are processes that yield compounds that influence and condition the organoleptic characteristics of wines and thus determine their quality. Consequently, the microorganisms responsible for biotransformation—bacteria and yeasts—have been extensively investigated with the goal of optimizing microbial metabolism for industrial purposes (217). The occurrence of killer yeasts in spontaneous wine fermentation has been examined. Killer yeasts were found in 88% of the fermentation processes of the wineries studied, and their prevalence was influenced by the fermentation stage and vintage period (408). *S. cerevisiae* is the predominant fermentative yeast because the chemical composition of the must substrate is more favorable for the growth of yeasts than for the growth of schizomycetes (210). Environmental parameters, however, such as temperature, oxygenation, pH, and, in particular, microbial antagonism, can markedly modify culture conditions and the development and success of alcoholic fermentation (49, 218). Historically, wine is produced by spontaneous fermentation by yeasts such as *Kloeckera*, *Metschnikowia*, and *Hanseniaspora* spp., which occur naturally on grapes and in the must. To overcome problems of unstandardized conditions, the use of pure cultures of enologically selected species, such as *S. cerevisiae*, with known and desired genetic characteristics has been adopted (269). Killer-resistant strains of *S. cerevisiae* isolated from spontaneous fermentations in different vineyards in several countries were characterized by using spheroplast killing and karyotyping. Almost all (92%) of the strains derived from the K2 killer toxin-producing wine strains from the same fermentation could be grouped into four karyotypes. The remaining strains were killer resistant at the cell wall level and were genetically unrelated (59, 167, 180, 413). The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programs has been demonstrated to identify and control industrial strains and to differentiate hybrid and parental strains (78, 384, 410). Yeast genetic manipulations may provide new transformed strains that, as well as possessing relevant wine-making characteristics such as high fermentation rates, ethanol production, SO_2 resistance, production of β-1,4-endoglucanase, flocculation, and favorable aroma, also possess the killer phenotype to favor their antagonism with indigenous microbiota (29, 73, 89, 103, 157, 169, 309, 321, 324, 348, 353, 414). This goal has been approached over the years by using classical genetic methods such as hybridization through matings among spores or haploid cells, newer biotechnology procedures such as somatic hybridization by fusion of protoplasts induced by chemical or physical procedures, and transformation through introduction of foreign DNA by plasmids (100, 304). A stably inheritable killer phenotype in the industrial *S. cerevisiae* killer system has been obtained by electroinjection of dsRNA from a superkiller strain into distillery and brewery recipient strains (320). Fusion between UV-killed protoplasts of *S. cerevisiae* with the killer phenotype and live protoplasts of the highly flocculent strain of *S. cerevisiae* in the presence of polyethylene glycol led to the construction of highly flocculent fusants with a killer character that provided an improved industrial yeast fermentation (182). Novel hybrid K2- and K3-type killer *Saccharomyces* wine yeasts were constructed by the protoplast fusion technique. The quality of wine produced with the hybrids was similar to that made with the parental strains, even though the stability of killer activity was greater for the parental strains than for the fusants. The killer properties of the fusants, however, proved to be sufficient to suppress undesirable contaminating yeast strains at the start of the fermentation processes (103, 185, 247). Conversely, in studies of the influence of curing the killer phenotype in *S. cerevisiae* wine strains by acridine orange treatment, the number of cells per milliliter at the beginning of the fermentation and in the exponential phase of growth was greater in grape juices inoculated with cured strains than in those inoculated with the isogenic counterparts, as were the level of CO_2 production and the rate of fermentation (58, 59). In the fermentation of wine by *S. cerevisiae*, the pH values of the must (3 to 3.5) should minimize the relevance of K1 toxin in comparison with K2 toxin, and, significantly, the K2-type *S. cerevisiae* strains are mostly rescued from wine production (180, 258, 411).

The wide occurrence of the killer phenotype in yeasts of enological interest that have been isolated in several countries has increased interest in their use in wine fermentation processes. Strain compatibility and the type of associated yeast strains that can be used in the alcoholic glucose and xylose fermentations by the coculture process should be carefully evaluated to avoid interference between killer and susceptible cells (219). A long debate has centered on the initial ratio between killer and susceptible yeast cells that is needed for former to predominate. Experimental studies have shown that the killer yeast predominates when it is prevalent or at least equivalent in number to susceptible yeasts at the time of inoculation. Determination of the killer yeast activity in fermenting grape juice by using a killer *S. cerevisiae* strain marked with the *Escherichia coli* β-glucuronidase gene and cured of its M-dsRNA has shown that killer activity was detectable only when the ratio of killer to susceptible cells exceeded 1:2, while at the highest ratio of killer to susceptible cells tested (2:1), complete elimination of susceptible cells was not achieved (275). However, it has been found that during the fermentation process, the killer effect is detectable at lower concentrations of the killer cells up to 1:500 (156, 157, 164, 306, 403, 411). Killer yeasts that naturally contaminated the must drove the fermentation that had been started initially by susceptible yeasts, even though some undesired effects occurred (164, 180, 411).

The limitation of killer toxin production by high sugar concentrations has been questioned even though, in controlled fermentations, killer effects have been reported to occur under those conditions (352, 403, 411). The use of killer yeasts as starters in wine fermentation processes has been reported to be important in protecting the final product from refermentation and film production. The killer yeasts inhibit the indige-

nous microbiota responsible for those undesired phenomena (156). The potential relevance of the killer phenotype in yeasts used as starters for fermentation has led to selection of the most appropriate isolates for avoiding the growth of contaminating yeast strains that usually belong to the genera *Candida*, *Hanseniaspora*, *Kloeckera*, *Pichia*, and *Saccharomyces* (other than the starter strain) (160, 163, 306, 307). The use of a killer *S. cerevisiae* isolate that has toxic activity restricted to strains of the same species appears to have limited value (453). To overcome this limitation and on the basis of the known killer-susceptible interactions that occur between different yeast genera and species, studies have been performed to engineer a superkiller yeast characterized by multiple killer factors and able to prevent contaminations by taxonomically unrelated yeasts (275, 348). By using a procedure of simultaneous protoplast fusion, a novel *S. cerevisiae* killer strain with the desirable killer phenotype was constructed and further used for the transfer of that character into a commercial wine yeast (379). Conversely, the use of neutral (nonkiller and nonsusceptible) yeasts in place of killer yeasts has been proposed for fermentation processes, since the latter could contaminate the environment, thus making difficult the further use of susceptible starter yeast strains (411).

Before selecting a yeast starter strain for fermentation, the susceptibility of the indigenous microbiota to killer strains should be carefully evaluated. Knowing that killer phenotypes are widespread, the yeast starter should also be a killer or at least a neutral strain but not a susceptible strain, to avoid the failure of the fermentation process. Conversely, when susceptible phenotypes predominate in the indigenous microbiota, the need for a killer yeast starter becomes less important. In the absence of information on the behavior of the indigenous microbiota, a killer starter would still be preferred. It has been postulated that the ideal candidate for an inoculum starter would be an engineered yeast resistant to microbial toxins and producing a toxin lethal to indigenous yeasts, molds, and bacteria (304).

Also of potential industrial interest is the finding of osmophilic killer yeasts, whose toxic activity was demonstrated only in the presence of high concentrations of salts such as NaCl or KCl (190, 380, 381). *Kluyveromyces* strains with killer activity against *Z. rouxii* in the presence of salts were screened to detect isolates useful in developing natural preservatives to prevent refermentation of salted fermented foods (208). Killer spectra were also investigated to select killer yeasts, such as *K. lactis*, that might inhibit the growth of wild yeasts, potentially causing aerobic deterioration of silage. Crude killer toxin produced by *K. lactis* was characterized by rapid killing of a wide spectrum of susceptible target yeasts and inhibited the growth of *S. cerevisiae* in both liquid and solid media, particularly when lactose was used as the carbon source in place of glucose (200). Killer yeasts such as *P. anomala*, *P. guilliermondii*, and *S. cerevisiae* have been used in the biocontrol of mold growth in high-moisture wheat stored under airtight conditions. The wheat frequently becomes contaminated by mycotoxigenic *Aspergillus* and *Penicillium* species (276). The remarkable interest of the brewing industry in using killer yeasts to prevent the growth of competing susceptible yeast strains indicates a potential value of killer toxins for use in the prophylaxis of fungal diseases (454).

Opportunistic Killer Yeasts

In recent years, opportunistic yeasts have gained increasing medical importance, particularly in immunocompromised hosts. Apart from the dramatic prevalence of *C. albicans* and *Cryptococcus neoformans* infections, rare species of yeasts, including killer yeasts from species such as *Kluyveromyces*, *Pichia*, and *Saccharomyces*, have attracted attention. *P. anomala* has proven to be the etiologic agent of several clinical cases of infection with outcomes ranging from death to recovery (159, 243, 255, 256, 263, 347, 423). The yeast infections were clearly related to such underlying predisposing factors as low birth weight, hematologic problems, malignant neoplasia, intraventricular hemorrhage, bronchopulmonary dysplasia, congenital heart disease, necrotizing enterocolitis, multiple sclerosis, intravenous drug abuse, acute promyelocytic leukemia, catheter-related fungemia, renal transplantation, immunosuppressive therapy, total parenteral nutrition, and multiple congenital abnormalities. Even though documented cases are few, the reports of *P. anomala* infections should be considered conservative since most of the infections caused by this species may be neglected or unreported by the large majority of investigators. An outbreak of colonization and infection with *P. anomala* has been reported in a neonatal intensive care unit, where 52 babies were found to be infected (256). In particular, eight premature babies receiving ventilatory support, one of whom was demonstrated to have been previously colonized by the yeast, had multiple problems associated with low birth weight and infections, such as fungemia and/or ventriculitis, and were admitted to the intensive care unit. A *P. anomala* infection contributed to death in one case. While *P. anomala* appeared to be the sole pathogen isolated, specific antibodies were detectable in most of the diseased babies a few months after the infection. In this study, despite in-depth investigations of hospital personnel and the environment, the babies appeared to be the most probable reservoir of infection (256). Surveillance of blood culture yeast isolates has shown that the incidence of nosocomial fungemia caused by potential killer yeasts, such as *P. anomala* and *S. cerevisiae*, is increasing. These infections are often associated with high mortality rates. The role of *S. cerevisiae* and other potential killer yeasts as human pathogens with particular reference to fungemia and AIDS patients has been reviewed (6, 141, 322, 390, 391, 444).

In Vivo Production of Yeast Killer Toxins

The recognition of human colonization and infection by killer yeasts should elicit further considerations of the importance of killer toxins in yeast pathogenicity. On the assumption that mycoses are caused by fungi according to a mechanism comparable to that involved in bacterial infections and mainly referable to toxigenicity and invasiveness, yeast killer toxins could play an important role in the host tissues by inhibiting susceptible biological competitors. Microbial toxigenicity, such as for the bacteriocins, may be of some relevance in the regulation of microbial dynamics and in the in vivo modification of ecosystems such as exist in the gut and mouth (234). The regulation of the gut microbiota by incorporation of living microorganisms in foods (probiotics) with the goal of promoting a selected colonization of the gastrointestinal tract has been pursued. In this regard, a bioregulatory effect of selected microorganisms by their interactions with enteropathogenic competitors, especially the lethal effect of killer yeasts on *E. coli*, has been proposed (131).

Competition experiments with microorganisms in gnotobiotic animals or studies evaluating the success of colonization of animal or human body sites have suggested that the toxigenic properties of microorganisms may displace or inhibit the establishment of biological competitors such as the resident microbiota (36, 97, 113, 177, 343, 424). Toxin production by toxin-producing microorganisms has also been demonstrated

in the course of experimental infections of laboratory animals (37, 77, 150, 152, 294, 351). Clinical and epidemiological surveys have indicated that toxigenic microorganisms, characterized by low pathogenicity, may prevent the colonization of more virulent pathogens (7, 323). Since a low prevalence of killers has been noted among pathogenic yeasts, with the exception of *Candida*, *Cryptococcus*, and *Torulopsis*, and no one has as yet surveyed the yeast forms of the dimorphic pathogenic fungi for killer toxin production, no apparent relationship has been found to exist between killer phenotype and pathogenicity (46, 317, 374). The first report of killer toxin production by an opportunistic yeast was in *Candida* (*Torulopsis*) *glabrata* (46). It was given the acronym PEST: pull effect stimulating toxin. Other toxins were found and placed in several classes in *C. glabrata* on the basis of their killing specificity (191).

Canditoxin has been detected by the fluorescent-antibody technique in the kidneys of mice experimentally infected with a *C. albicans* toxin-producing strain (178). Histological studies carried out on normal and immunosuppressed mice infected with *P. anomala* killer toxin-producing strains revealed tissue proliferation and invasiveness independent of the immunosuppressive procedures adopted. Yeast killer toxin was detected as fluorescence on the outer part of the parasitizing yeast killer cells by using specific anti-yeast killer toxin monoclonal antibodies in the indirect immunofluorescence assay. Immunoreactivity appeared stronger in the older parts of the mycelium than in the budding cells (294). This feature confirmed the mode of toxin secretion by *P. anomala* determined in vitro with the same anti-yeast killer toxin monoclonal antibodies. Significantly, no fluorescence could be detected in the tissue cells surrounding the proliferating yeast cells and mycelium, suggesting that host cells were not involved in yeast killer toxin interactions, even though accurate procedures for the preservation of tissue cell receptors were not used.

Although the in vivo possible production of yeast killer toxin was demonstrated, its relevance to the mechanism of infection associated with killer yeasts, its involvement in pathogenicity, the virulence of toxinogenicity itself, and the elimination of the susceptible resident microbiota remain questionable. Further comparative studies on the interaction of the killer yeast *P. anomala* with susceptible and natural toxin-resistant mutant strains of *C. albicans* performed under various conditions have shown that killer inhibition may occur in vitro at pH values and temperatures that are not encountered in vivo. Under adverse growth conditions, moreover, the *P. anomala* killer yeast was still able to produce a toxoid that was unable to kill recognized susceptible strains but was antigenically related to the active or heat-inactivated killer toxin, as demonstrated by their homologous reactivity with a specific monoclonal antibody (74). These findings suggested that *P. anomala* killer toxin may not be involved in vivo as a potential virulence factor or be in competition with other potentially susceptible microorganisms for colonization in the course of animal and human infections. Inhibition, moreover, was shown to occur, even in vitro, only by using large amounts of preformed killer toxin instead of the secreting killer yeasts, since, under experimental conditions, the overgrowth of the killer yeast proved to overwhelm the killer effect.

Experimental Enterotoxicity of Yeast Killer Toxins

The potential relevance of the preformed killer toxin produced by *P. anomala* has been demonstrated by the induction of secretion and severe acute injury in an isolated rat small intestine model (277). Intestinal fluid homeostasis and electrolyte balance were heavily affected by the *P. anomala* killer toxin when a standard perfusion technique was used. No significant changes in water and electrolyte secretion were observed when heat-inactivated killer toxin was used in the same model. Histological examination of most of the toxin-perfused jejuna showed ischemic degeneration of villi and sloughing of surface epithelium. Since no specific receptor for killer toxin could be presumed to exist from immunofluorescence studies with specific monoclonal antibodies, the mechanism of induction of gastrointestinal symptoms and damage of the intestinal wall in humans which has been associated with *P. anomala* infection remains to be elucidated even in the presence of active and preformed yeast killer toxins. Colonization of the human gastrointestinal tract by fungi is abundantly documented, and the related enteric symptoms are well characterized (60, 93, 98). It is known, indeed, that toxins produced by enteric microorganisms can alter normal secretory and absorptive intestinal processes by selective destruction of normal absorptive cells or, alternatively, by stimulation of normal secreting pathways or inhibition of normal absorption to cause abnormal net secretion (106, 107, 151, 192). Conversely, no effect of a killer toxin produced by a strain of *S. cerevisiae*, a yeast used in beverage production, was observed in several animal model systems, even when the yeast was present at concentrations higher than expected in the beverages. In addition, because of its lability, *S. cerevisiae* killer toxin is unlikely to represent a hazard when consumed orally (279).

Interferon Induction by dsRNA Yeast Killer Plasmids

Fungal viruses (mycoviruses) were originally observed in diseased mushrooms. The finding of interferon induction by and, thus, antiviral activity of extracts such as statolon from apparently healthy fungal isolates such as *Penicillium stoloniferum* and *P. funiculosum* increased interest in this distinct area of virology. The antiviral activity was related to the presence of dsRNA, similar to the occurrence of satellite dsRNA in killer yeasts such as *S. cerevisiae* and *U. maydis* (11, 172, 201). Yeast dsRNA proved to be a potent inducer of interferon, although, despite its efficacy as an antiviral agent, it was too toxic in experimental animals to be used in clinical trials on humans (82, 274). In recent years, all of the interferon types have been purified to homogeneity, their genes have been cloned, and plentiful supplies of interferon have been produced by recombinant DNA technology in bacteria and yeasts. Yeast dsRNA continues to be important in interferon research because, as well as inducing interferon synthesis, it is essential for the activity of the enzymes involved in interferon action, a mechanism for ensuring that these enzymes are switched on only during viral infection (274).

EPIDEMIOLOGY BY YEAST KILLER SYSTEMS

Reevaluation of the Yeast Killer Phenomenon

Since the original observation of microbial antagonism reported by Pasteur (271), inhibitory interactions have been shown to be a universal feature among microorganisms. One of the most important criteria is that microbial interactions usually occur within members of the same or congeneric species. Differential intraspecific susceptibility of microorganisms to microbial products presumably is referable to nuclear mutations to resistance selected during evolution. The resulting discriminatory phenotypes have been used extensively for biotyping procedures. The need to discriminate among fungal strains within a species, particularly for isolates from immuno-

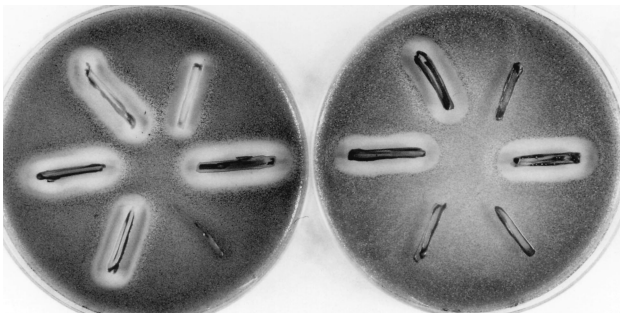

FIG. 6. Differential inhibitory effect of streaked killer yeasts against two strains of *C. albicans.*

compromised patients in the hospital environment, has dramatically increased during the past few years (285). Analogous to the application of bacterial susceptibility to bacteriocins and bacteriophages for use in typing procedures, yeast killer toxins may serve as specific markers in epidemiological surveys (146, 153, 186, 225, 227). Simple and low-cost typing systems may be developed based on the susceptibility to or the production of yeast killer toxins, particularly when used in association with other procedures such as serotyping or susceptibility to chemicals (221). As in bacteria, intraspecific differentiation of fungal strains by phenotypic properties, even though less reliable than biotyping procedures based on genotypic properties (e.g., DNA fingerprinting or electrokaryotype), may still prove useful when a large number of isolates must be investigated, particularly in laboratories of the third-world countries with economic and technical limitations (17). Biotyping techniques, based on yeast killer systems, may be performed by either direct or indirect procedures. By using purified and quantified killer toxins in place of killer yeasts, culture conditions that could be incompatible with killer toxin production or growth of the test organism are avoided (145, 286, 453). New rapid assays for evaluating yeast killer toxin activity, particularly of *S. cerevisiae*, have been developed based on the bromocresol purple fluorescent test. This test can be used to determine the number of yeast cells with a damaged plasma membrane and has proved to be as precise as the conventional plating test and more sensitive than the well test (214, 216).

The surprising recognition that a number of killer yeasts, particularly belonging to the genera *Pichia* and *Williopsis*, may display their toxic activity in the classic assays not only against isolates of the same species but also against lipophilic yeasts, pathogenic molds, bacteria, aerobic actinomycetes, and achlorophyllous microorganisms has led to the extension of the concept of the yeast killer phenomenon and the applications of the yeast killer system as well (288).

Strain Differentiation by the Yeast Killer System

The first application of the yeast killer system for the simple intraspecific differentiation of pathogenic fungi was reported for *C. albicans* isolates (Fig. 6) (284). The potential for strain discrimination could be greatly improved by using a larger number of effective killer yeasts or their purified toxins in the indirect procedure (61). The use of the yeast killer system as an epidemiological tool proved to be of great value in identifying presumptive cases of hospital-acquired fungal infections (285). The same yeast killer system proved to be very valuable when applied to the differentiation of opportunistic yeasts other than *C. albicans* such as *C. glabrata*, *C. kefyr*, *C. parapsilosis*, *C. tropicalis*, and *Cryptococcus neoformans* (251). In the indirect procedure for biotyping, killer toxins are used in place of killer yeast cells for strain differentiation of *C. albicans* isolates (Fig. 7). By using a computer-aided system programmed to evaluate, record, and compare the halo of inhibition displayed by each killer toxin on each susceptible strain, the *C. albicans* isolates were also grouped into biotypes within a predefined margin of error (286). Computer analysis of data might avoid subjective interpretation of the results, while the recorded data permit comparison of new results with the groups previously coded, thus simplifying the application of the system to epidemiological studies. Evidence that selected killer yeasts may display their inhibitory effect on different species of molds other than yeasts prompted evaluation of the potential of the yeast killer system to differentiate strains of *Pseudallescheria boydii*, *Aspergillus niger*, and *Sporothrix schenckii*. The demonstration of different biotypes within mycelial fungi proved that the killer system represented a feasible and simple method for epidemiological studies of microorganisms that are difficult to approach by other procedures (289). The opportunity to differentiate members of the genus *Aspergillus* was of great value for studying the impact of the airborne opportunistic fungal pathogen *A. fumigatus* in hospitalized patients. The study was conducted in the course of a clinically suspected outbreak of aspergillosis that in some cases was proven by postmortem histological examinations (101).

The extension of the killer system to strains of commercial value, such as *Penicillium camemberti*, a mold used in industry for cheese fermentation, would permit ready detection of specific strains and control of their culture conditions (289).

Microbiologists have become increasingly aware of the need for epidemiological markers for infections caused by slowly growing bacteria such as the mycobacteria and the aerobic actinomycetes. While strain differentiation of mycobacteria has been accomplished by serological or phage-typing methods, the etiological agents of nocardiosis remain to be differentiated, even though a serotyping method was applied in an outbreak of *Nocardia asteroides* infection to correlate patient isolates with those from the environment (75, 186, 281, 385, 386). Serotyping implies the availability of specific antisera that might not be commonly available in laboratories. In contrast, the yeast killer system has been shown to provide a feasible and reproducible method to differentiate isolates of *N. asteroides*, *N. brasiliensis*, *N. otitidis caviarum*, and *Actinomadura madurae* (252).

The yeast killer system has proven to be fruitful not only in the differentiation of other important slowly growing pathogenic bacteria, such as the mycobacteria, but also in the differentiation of faster-growing gram-positive and gram-negative bacteria (253). When used to investigate the serotypes of bacterial isolates, the yeast killer system was able to differentiate

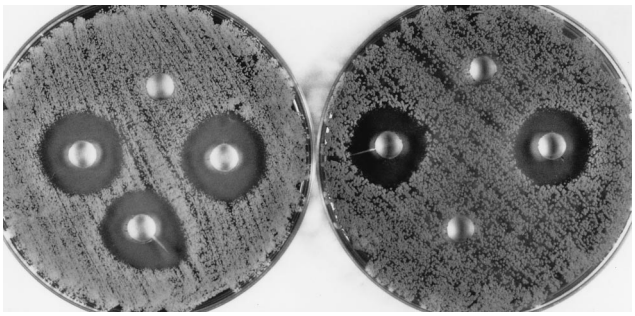

FIG. 7. Differential inhibitory effect of yeast killer toxins against two strains of *C. albicans.*

isolates of *Neisseria meningitidis* group C. Many different phenotypic or genotypic systems, such as phage typing, enzyme production, bacteriocin production or bacteriocin susceptibility, DNA hybridization, and resistance plasmid analysis, have been used for biotyping bacterial species (2, 13, 126, 181, 195, 225, 235, 254, 355, 382, 401). The killer system, properly adapted to the growth requirements of the potentially susceptible bacteria, may represent a common and relatively simple method for studying large numbers of isolates by small laboratories. Identification of the growth requirements of the investigated microorganisms was essential to determine their potential susceptibility to yeast killer toxins, even though commercial media were used to simplify the procedure. Interestingly, the killer yeasts displayed their toxic activity under remarkably different conditions of pH, temperature, and oxygen concentration.

The importance of the effect of the growth conditions on the test results was strikingly evident in a study of anaerobic yeast killer systems (299). Anaerobiosis influenced the activity of the purified killer toxins of several yeasts from different genera on yeast isolates known to be susceptible to the same killer yeasts under aerobic conditions. The killer phenomena were affected differently by the anaerobic conditions. For some purified killer toxins, anaerobiosis did not change the effect on any of the susceptible strains studied, whereas for others, activity against only certain susceptible yeast isolates was lost under anaerobic conditions. Thus, in some cases, anaerobic conditions interfered more with the metabolic processes of potentially susceptible strains than with toxin production by the killer yeasts. The use of a panel of selected killer yeasts that exerted their activity against known susceptible yeast strains under anaerobic conditions in a medium compatible with the growth requirements of anaerobes permitted the use of the killer system for biotyping of *Bacteroides fragilis* for epidemiological purposes. This anaerobic microorganism is the one most commonly associated with anaerobic infections (110). The extension of the yeast killer phenomenon to anaerobic conditions revealed the adaptability and expanded potential of the yeast killer system as an epidemiological tool.

Species and Variety Identification by the Yeast Killer System

The yeast killer system, when properly used, has proved to be of great value in the identification of the species and varieties of heterogeneous microorganisms. On the basis of previous studies that permitted differentiation of strains belonging to species of aerobic actinomycetes, the yeast killer system was used to separate species that had been identified as *N. asteroides* complex (115, 252). Different and complementary approaches, such as antigen-induced hypersensitivity, DNA-DNA hybridization, and antibiotic susceptibility tests, supported separation of the *N. asteroides* strains into *N. asteroides* sensu stricto and *N. farcinica* (140, 213, 232, 328, 405). Additional numerical studies provided information for the further taxonomic subdivision of *N. asteroides* sensu stricto into *N. asteroides* new sense and *N. nova* (406). Conventional methods of identification based on patterns of substrate decomposition and acid production from sugars are unable to discriminate among these three presumptive species, which were generally considered to be members of the *N. asteroides* complex (422). Differentiation into species, however, is as important as epidemiological, pathogenetic, and antibiotic susceptibility patterns that result in differences in the management of patients. The clinical outcome of patients appears to be clearly related to the identity of the species of their infectious agent (25, 80, 421, 422). The combined use of only two selected killer yeasts exerting a differential killer activity against randomly selected clinical isolates belonging to each species of the *N. asteroides* complex provided a perfect correlation with the most sophisticated methods of identification. This work established the yeast killer system as a rewarding tool for the classification of taxonomically heterogeneous species of microorganisms (305).

Susceptibility to yeast killer toxins specific for the *gattii* and *neoformans* varieties of *C. neoformans* was revealed by using a killer yeast belonging to the species *Cryptococcus laurentii* (138). The simple and economical killer assay may allow the identification of the two varieties regardless of the availability of specific and more expensive antisera.

THERAPEUTIC POTENTIAL OF YEAST KILLER SYSTEMS

Antibiotic Properties of Yeast Killer Toxins

Natural antibiotics are low-molecular-weight substances, usually produced by bacteria and fungi, that may interfere with enzymatic functions by acting as analogs or binding to and altering cellular targets (24, 114). Yeast killer toxins show a high specificity of action since the producing strains are immune. Thus, the killer factor effectively acts as a specific fungal antibiotic, implying that specific surface receptors are present. The search for alternative antifungal agents can be based on the assumption that sites specific for a killer factor have other essential functions whose inhibitors could be antibiotics (47). Even though it might appear improper to consider the results of microbial interactions to be related to the activity of conventional antibiotics, microbial antagonism may represent the rationale for the design of new antibiotics based on the selective pressure of natural evolution. If the competitive advantage of the killer phenotype appears to be obvious, the biological reason why the susceptible phenotype has been maintained unaltered during evolution seems to be inexplicable (145). Although microbial toxins may be compared to classic peptide antibiotics, they differ in binding to the specific surface receptors of susceptible microorganisms. These receptors must be physiologically essential because they have been preserved despite the handicap of susceptibility to the lethal effect of toxins secreted by antagonistic microorganisms. Thus, a yeast killer toxin-derived antibiotic that targets essential and physiological cellular structures or functions but is not toxic to higher eukaryotic cells would be of great value.

Yeast killer toxins are themselves proteins that are too large to be used in systemic antifungal therapy without eliciting an immune response. If they could be delivered without encountering the immune system, they might prove useful if they do not have adverse effects on human cells. The high prevalence of the susceptibility of pathogenic yeasts to killer toxin produced by certain yeasts, particularly belonging to the genera *Pichia* and *Williopsis*, could lead to practical applications in the design of synthetic derivatives to be used as antifungal agents.

The 10.721-kDa toxin, produced by *W. mrakii*, was found to inhibit selectively β-glucan synthesis in the cell wall of susceptible yeasts by a mechanism similar to that of antifungal antibiotics such as aculeacin A, echinocandin B, and papulocandin B (447). These agents also inhibit the in vitro activity of β-1,3-glucan synthetase of yeasts (445). These compounds are considered to be the most important structural elements of the yeast cell wall. Thus, their inhibition may render the wall somehow osmotically fragile or defective, resulting in the death of the yeast cell. Unlike other yeast killer toxins, the one produced by *W. mrakii* proved to be highly stable, and this prop-

erty made it possible to examine its antimicrobial activity against a wide variety of opportunistic fungi. A conventional MIC assay was used under conditions similar to those adopted for conventional antifungal agents (447). Interestingly, differential activity of four selected yeast killer toxins on the mycelial and yeast forms of four isolates of the dimorphic fungus *Sporothrix schenckii*, a well-known agent of subcutaneous mycosis, has been reported (292). The results confirmed that the yeast killer phenomenon occurs for both morphological forms of the fungus, which are susceptible to the activity of the same killer toxin. The apparent uniformity of susceptibility implies the existence of common cell wall receptors during the molecular and cellular events that accompany the morphologic variations in the dimorphic fungi and affect the susceptibility to antibiotics (16, 242). The finding is remarkable because therapeutic approaches to the treatment of systemic mycoses should be applied to the tissue form that characterizes the etiologic agents in host tissues.

Therapeutic Properties of Yeast Killer Toxins

Even though it might be presumed that only conventional antibiotics should be used as effective therapeutic agents, despite the induction of resistance in susceptible microorganisms, attempts have been made to use yeast killer toxins in the treatment of experimental fungal infections. To avoid possible protease inactivation and alteration of the stability of the molecules at physiological pH and temperature, a model of an experimental superficial mycosis has been evaluated (287). The extension of the yeast killer phenomenon to taxonomically unrelated microorganisms, including *Malassezia furfur* and *M. pachydermatis*, led to the demonstration of the therapeutic effectiveness of a killer toxin produced by a selected strain of *P. anomala* (ATCC 96603) in experimental pityriasis versicolor-like lesions and otitis externa in laboratory animals (287). Pityriasis versicolor is a superficial mycosis that appears to be a suitable model for the new therapeutic approach of topical administration of a crude yeast killer toxin. However, the potential therapeutic effect of the purified active principle to be administered by the systemic route for the treatment of deep-seated infections should be evaluated, particularly with regard to the instability of the molecule at physiological temperature and pH.

SEROLOGY OF YEAST KILLER SYSTEMS

Antigenicity of Yeast Killer Toxins

The most important consideration in the use of yeast killer toxins as therapeutic antifungal agents is the possible induction of side effects. Aside from toxicity, immunogenicity could affect the use of killer toxins for the treatment and prophylaxis of fungal infections despite their wide spectrum of antimicrobial activity and potential therapeutic effect exerted through topical application. Thus, because they are foreign glycoproteins with high molecular mass, yeast killer toxins will not be readily useful as antibiotics because of their strong antigenicity. Anaphylactic reactions or neutralizing antibodies might be evoked by the immune system after administration of yeast killer toxins. It should be borne in mind that only the small biologically active killer peptides, devoid of immunogenicity, could have therapeutic use. In this regard, the immunogenicity of yeast killer toxin might be advantageous in the production of specific monoclonal antibodies that could be used for the one-step purification of the toxin by affinity chromatography. Monoclonal antibodies could also allow peptide mapping of these purified yeast killer toxins for use in the synthesis of artificial peptides that retain their biological activity but are devoid of immunogenic and toxic properties.

Monoclonal Antibodies against Yeast Killer Toxins

From serological cross-reactivity of yeast killer toxins with monoclonal antibodies, one might presume that they evolved from the same ancestor molecules analogous to the similarities in their sequence of amino acid regions. Monoclonal antibodies that possessed both binding and neutralizing activities were raised against the heat- and pH-stable basic killer toxin of *W. mrakii*. The antibodies were used for purification by affinity chromatography (446).

Monoclonal antibodies were produced to purify a killer toxin produced by *P. anomala* ATCC 96603 because conventional chemical procedures were not feasible for large-scale production. One monoclonal antibody (MAb KT4) neutralized the activity of the killer toxin in vitro against a susceptible *C. albicans* reference strain and was successfully used to characterize the killer toxin (290). Immunodetection might be essential for understanding the synthesis of the yeast killer toxin, its cytoplasmic transport by cellular organelles, and its passage across the plasma membrane before its secretion through the cell wall.

Killer toxin localization at the cell surface of the *P. anomala* ATCC 96603 yeast cells was studied by an immunofluorescence assay (IFA) with MAb KT4 on living cells. A "punctated" fluorescence covered the surface of almost all mature blastoconidia and appeared as dashes. A more intense linear labeling appeared on a great number of cells; this pattern corresponded, in most of the cases, to their bud scars. Different intensities of surface labeling provided morphological suggestions of an organized secretion of the *P. anomala* killer toxin by preferential pathways through defined regions of the cell wall (55).

Indirect immunogold electron microscopy was performed with MAb KT4 on ultrathin sections of *P. anomala* cells. Cytoplasmic labeling was observed with no significative fixation in the nuclei, mitochondria, or large vacuoles. Recognition by MAb KT4 of cytoplasmic epitopes could be referable to the intracellular localization of protoxin or mature killer toxin molecules. Moreover, a prevalent localization of gold particles in the cell wall was proven by the use of different procedures, in agreement with observations made by IFA (55, 57).

Serological Analysis of Yeast Killer Toxins

Experiments carried out by double immunodiffusion showed that MAb KT4 produced homologous precipitin bands in the reaction with the killer toxin produced by *W. mrakii*. Conversely, yeast killer toxins of various species and genera (*Candida*, *Kluyveromyces*, and *Saccharomyces*), known to be encoded by different genetic determinants, have proven to be serologically unrelated in tests with MAb KT4 both in a double immunodiffusion procedure with the secreted toxin and by the indirect immunofluorescence assay with the whole toxin-secreting cells. Significantly, MAb KT4 proved to be specific for killer toxins produced by members of the genus *Pichia* by both procedures. The differential immunological reactivity of the killer toxins from different yeast systems demonstrates a clear antigenic heterogeneity (293). Antigenic heterogeneity, however, was also demonstrated within species belonging to the genus *Pichia*, formerly known as *Hansenula* (212). Differential toxinogenesis was evaluated by indirect immunofluorescence on whole cells and by double immunodiffusion on their metabolic products by using MAb KT4 (296). Even though the killer

phenotype of all of the *Pichia* isolates investigated was tested by their capacity to kill each other, the results verified the genomic variability for the same character within the same genus. Interestingly, the yeast killer toxins produced by several isolates of *Pichia* belonging to different species produced homologous precipitin bands in the double-immunodiffusion reaction with MAb KT4. The results obtained by this procedure and by indirect immunofluorescence were identical. Significantly, some *Pichia* isolates that proved to be reactive with MAb KT4 did not show activity in the reciprocal killer susceptibility test, an observation that could be due to the use of an improper susceptible strain. Conversely, other *Pichia* isolates with demonstrated killer activity did not react with MAb KT4, suggesting that different killer toxins may be produced by the same yeast strain. As reported for many other yeast systems, the *Pichia* isolates could manifest their killer or susceptible phenotype independently and according to the complementary yeast tested.

IDIOTYPY OF YEAST KILLER SYSTEMS

Origin of the Antibody Repertoire

Nature has granted an evolutionary plasticity to model antibody-combining sites around different antigens. The conformational structures of the variable regions of the heavy and light immunoglobulin chains are specified by chromosomal genes. The partition of these genes into segments is fundamental for the production of an enormous number of innate antibody-variable regions by two mechanisms: gene recombination and junctional diversity. Three classes of gene segments, V (variable), D (diversity), and J (joining), participate in genomic rearrangements to form the variable region of the immunoglobulin H-chain (V_HDJ_H) and L-chain ($V_\kappa J_\kappa$, $V_\lambda J_\lambda$) genes. Approximately 100 different V_H and V_L and 6 J_H and J_L segments, as well as 4 D segments, are known in the human genome, for a total of $>10^3$ genes. From this natural library, prior to the introduction of antigen, a variety of gene segments may be randomly selected by any developing B cell to encode the variable region of the antibody that it will secrete. Thereafter, the number of different antibody molecules that can be generated is $>10^6$. During the process of recombination, some imprecision may occur in the junctions between recombining V gene segments, contributing greatly to the variability of the system. The polypeptides produced by the newly aligned V_H and V_L gene fragments in each B-cell clone, the heavy- and the light-chain variable regions, fold over each other to create a unique three-dimensional structure, the antibody-combining site (paratope). Another source of immunoglobulin variance is the rate of mutation of V genes, the highest of any known gene. V gene somatic mutation in B cells, proliferating in the course of antigen-driven response, leads to the emergence of antibody variants tailored to the immunogen (233). B-cell clones, expressing immunoglobulins with the highest affinity for the antigen, are preferentially stimulated and expanded to dominate the antibody response.

The folds, crevices, and protrusions that constitute the three-dimensional surface of the immunoglobulin variable region contain many antigenic determinants, or idiotopes. Each antibody molecule carries a set of multiple idiotopes defining its private idiotype, which, in the course of the immune response, may induce the production of anti-variable region antibodies (anti-idiotypic antibodies or anti-idiotypes). It has been estimated that there are approximately 20 idiotopes in the V region of a single immunoglobulin molecule that may be distinguished by the use of corresponding monoclonal antiidiotopes. Antibodies might also have public idiotypes if different immunoglobulins are encoded by the same V_H gene segment. Thus, the same gene mutation affecting the combining site of an antibody without changing its idiotype can also result in the sharing of a public idiotype by antibodies with different specificities.

Idiotypes encoded by different V genes may have structures that are similar enough to react serologically with each other (cross-reactive idiotypes). The variable region of any antibody might be characterized phenotypically by its serologic signature (idiotype) or genotypically by its antigen-binding property (combining site). The two distinctive properties are not mutually exclusive, since an idiotype may occur anywhere within the combining site or in the variable region of an antibody (211, 268).

Idiotypic Network

The theory of the idiotypic network recognized that based on the dual character of the antibody molecule, the interaction of idiotypes and anti-idiotypes is intimately involved in the regulation of the immune system (183). According to the enormous number of potentially different variable regions, any antibody variable region (idiotype) should be matched by a complementary three-dimensional surface of another antibody variable region (anti-idiotype). There are profound implications in the idiotypic network theory. An idiotype produced in the course of the immune response against a particular antigen can stimulate the production of a complementary anti-idiotype (autoanti-idiotype). Production of antiautoanti-idiotypes might be self-limiting because of their similarity to the idiotypes. Immunological involvement of autoanti-idiotypes has been detected in a variety of clinical conditions (1, 91, 132, 133, 257, 314, 350, 377, 404).

Within the immune network, the definition of any antibody molecule is purely functional since idiotypes are supposed to behave like anti-idiotypes. In addition to B-cell receptors (the immunoglobulin idiotypes), the variable regions of the heterodimer receptors of T lymphocytes, the key regulatory elements of the immune system, should be theoretically considered. It would seem unlikely that B and T cell receptors reacting with the same antigen should have common idiotypes, because B-cell receptors recognize native epitopic structures whereas T-cell receptors process epitopes associated with class I or II major histocompatibility complexes. The existence of anti-idiotypic B and T cells that recognize different epitopes of the same idiotype, however, should not be disregarded to envision a B-cell network with the participation of anti-idiotypic T lymphocytes at any level. Since T lymphocytes can be of either the helper/inducer or suppressor variety, the potential exists for regulating the immune responses through idiotypic networks involving T cells.

The second implication of the network theory is that, among the three-dimensional shapes of millions of different variable regions, the combining site of anti-idiotypes, being complementary to that of the antigen-complementary idiotypes, may resemble the structure of apparently unrelated immunogenic determinants (102). The anti-idiotype variable region is likely to be the internal image of the external antigen and, in some circumstances, may mimic its biological activity (109, 134, 344).

Anti-Idiotypic Vaccination

The principle of idiotypic mimicry has suggested a new approach to immunoprotection since, by simulating a microbial antigen, an anti-idiotype could qualify as a surrogate vaccine (27, 261, 349). Anti-idiotypic reagents have been devised for

the manipulation of both B- and T-cell immunity to agents of viral (influenza, hepatitis B, AIDS) and parasitic (African trypanosomiasis, Chagas' disease, malaria, coccidiosis, lymphatic filariasis, onchocerciasis, schistosomiasis) diseases (196, 319). Anti-idiotypic immunization could have relevant advantages over conventional and DNA recombinant vaccination. Monoclonal anti-idiotypic vaccines would not contain any infectious agent, could be easily produced in large amounts, might direct the immune response against a single epitope that may not be immunodominant in the original immunogen (thus activating silent clones), could replace immunogenic determinants constituted by carbohydrates, and could induce a more effective T-cell response; finally, anti-idiotypes mimicking selected antigens might be properly engineered. The feasibility of using anti-idiotype-based vaccines for inducing immunoprotection against the in vivo toxicity of nonproteinaceous low-molecular-weight biological and chemical toxins has been verified. A murine monoclonal antibody protective against T-2 mycotoxin was generated and used to produce a second-generation monoclonal anti-idiotype that mimicked the tertiary conformation of T-2 toxin. Administration of the anti-idiotypic antibody to mice resulted in the production of a protective antibody against the toxicity of T-2 toxin. Thus, antibody-based vaccines may represent an exclusive strategy for vaccination against small nonproteinaceous toxins, such as ricin, whose extreme toxicity prevents their use as safe immunogens. Analogously, anti-idiotypic antibodies mimicking the inner-core region of lipopolysaccharide conferred immunoprotection against a lethal challenge with gram-negative bacterial endotoxin (66–70, 108, 230).

Anti-idiotypic antibodies have been envisioned for the immunotherapy of B-cell cancers that are characterized by presenting immunoglobulin receptors with a private idiotype at their surface (369). The specific binding of toxin-conjugated anti-idiotypes to such distinctive idiotypes may cause growth inhibition of tumor cells without affecting normal tissues (9, 209, 310). Anti-idiotypic antibodies bearing internal images of tumor-associated antigens have been used in approaches to the active immunotherapy of cancer (21, 165). Similar approaches may be used in other areas of medicine such as the prevention of graft rejection and allergies (23).

Yeast Killer Toxin Anti-Idiotypes

Conceptual homologies between the idiotype of an antibody molecule and microbial cell receptors may be envisioned (373). Both types of multifunctional structures contain recognition moieties capable of binding specifically either the antigen or the ligand, components responsible for signal transmission, and an effector part that is responsible for biological response. Complement fixation or interaction with Fc receptors may be induced by antigen binding, as is opening of ion channels in bacterial and yeast cell plasma membranes by bacteriocins and killer toxins, respectively (298).

Theoretically, the steric interaction between the cell wall receptor of a susceptible yeast strain and its specific yeast killer toxin may be comparable to the binding of the idiotype of an anti-yeast killer toxin monoclonal antibody and its complementary anti-idiotypic antibody (internal imagery of the yeast killer phenomenon). In this perspective, immunology may provide a unique tool for the study of yeast cell receptors and anti-receptors. The generation of monoclonal antibodies directed to biologically functional epitopes of yeast killer toxins should necessarily imply a steric homology between neutralizing antibody idiotypes and yeast cell wall receptors.

In light of the idiotypic network, anti-idiotypes performing like anti-receptor antibodies may be used to clarify the three-dimensional structure of killer toxin-specific cell wall receptors in yeast cells. Rabbits were immunized with hybridoma cells secreting MAb KT4 on the assumption that lymphocytes, bearing the proper idiotype as a receptor, might function as more effective immunogens. Rabbit antisera were shown to contain a subpopulation of anti-idiotypic antibodies sharing structural similarities with the functional epitope of the killer toxin binding to the cell wall receptor-like idiotype of MAb KT4 (291).

Anti-idiotypic antibodies complementary to specific receptors for the killer toxin might be useful reagents for elucidating structure-function relationships in yeast systems without isolation of the receptor (226). Anti-idiotypes could be effectively labeled with fluorescent dyes, radioisotopes, or colloidal gold particles for visualizing receptor sites very precisely on the surface of the presenting yeast cells. Phenotypic mutations and redistribution of receptors could be monitored under various physiological conditions in dynamic studies. The use of affinity chromatography-purified yeast killer toxin-like anti-idiotypic antibodies by indirect IFA permitted direct visualization of their interactions with the putative cell wall receptors on susceptible *C. albicans* cells. The specificity of the reaction was ascertained by the lack of IFA reactivity when anti-idiotypic antibodies were previously absorbed with MAb KT4. The degree of reactivity of the yeast cell wall outer layers varied according to the phase of growth. Immunofluorescence was detectable mainly in budding cells and germ tubes (295). The heterogeneous distribution of killer toxin cell wall receptors on cells growing differently could explain the higher susceptibility to *P. anomala* killer toxin encountered in yeast cells in their active growth phase. Putative yeast killer toxin cell wall receptors were also revealed by means of yeast killer toxin-like anti-idiotypic antibodies in immune *P. anomala* cells as well as in other yeast killer strains (*K. lactis* and *S. cerevisiae*) shown to be susceptible to the activity of the *P. anomala* killer toxin (297).

A consequent implication of the network theory is that anti-idiotypic antibodies, being the positive topochemical copies of the related antigens, might be expected to mimic not only their steric structure but also, in a positive fashion, their biological function. MAb KT4 affinity chromatography-purified anti-idiotypes were investigated for their in vitro killing activity against *C. albicans* cells susceptible to yeast killer toxin. CFU assays were performed comparatively by using equal inocula of yeast cells incubated overnight with anti-idiotypic antibodies previously absorbed with the complementary MAb KT4 and with phosphate-buffered saline as controls.

Analogous to methods for determining yeast killer toxin activity and its standardization, the fungicidal activity of anti-idiotypic antibodies was determined by viable counts and expressed in anti-idiotypic units per milliliter (216). Anti-idiotypes, which biologically mimic the activity of yeast killer toxin and then act as antibiotics, have been defined as "antibiobodies" (297).

Interestingly, the same experimental approach showed that yeast killer toxin-like anti-idiotypic antibodies were also able to kill *P. anomala* cells that had been previously recognized to be immune to the activity of their own toxin. The self-immunity systems that prevent yeast killer cells from suicidal tendencies were apparently bypassed by toxin-mimicking anti-idiotypic antibodies.

The neutralization of killer activity by absorption with MAb KT4 bearing a cell wall receptor-like idiotype attested to the specificity of the antimicrobial properties of yeast killer toxin-like anti-idiotypic antibodies. It may be hazardous to conclude that anti-idiotypic antibodies merely reproduce the activity of a

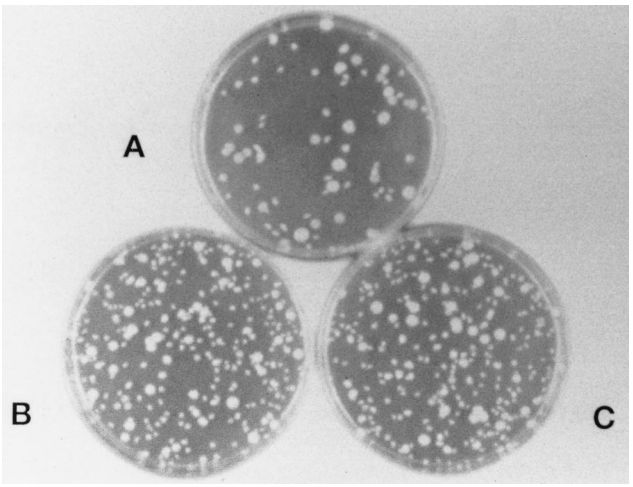

FIG. 8. CFU of standardized inocula of *C. albicans* cells treated with *P. anomala* killer toxin-like anti-idiotypic antibodies (KT-IdAb) from mice immunized by idiotypic vaccination with a killer toxin neutralizing monoclonal antibody (MAb KT4) (A), phosphate-buffered saline (B), or KT anti-idiotypic antibodies previously absorbed with MAb KT4 (C).

P. anomala yeast killer toxin for which an exclusive although still undetermined mechanism of action has been hypothesized (327). In susceptible cells, however, yeast killer toxin cell-wall receptors should account for the in vitro killing effect of anti-idiotypic antibodies, just as the receptor-like idiotype of the monoclonal antibody is concerned with the neutralization of the yeast killer toxin activity. Receptor binding, however, may be only a prerequisite for yeast killer toxin and anti-idiotypic activities. Signal transmission to the membrane and interior of the cell could require further components that differentially exert regulating and effector functions. The lethal effect of anti-idiotypic antibodies against killer cells immune to self-toxin is suggestive, however, of an independent antimicrobial activity.

Idiotypic Vaccination

The in vitro biological properties of anti-idiotypic antibodies may be reflected in vivo by immunization with the complementary monoclonal antibody (idiotypic vaccination). Parenteral administration of MAb KT4 to syngeneic mice resulted in a significant degree of protection against lethal intravenous infection with yeast killer toxin-susceptible *C. albicans* cells in comparison with unvaccinated or isotype-matched irrelevant monoclonal antibody-immunized controls (300).

The relationship of anti-idiotypic antibodies elicited in the serum of immunized animals with immunoprotection, as reflected by a significant increase in the survival time, was demonstrated by increasing titers during the course of immunization. Mouse anti-idiotypic antibodies were detected by an enzyme-linked immunosorbent immunometric assay that exploited the competition of the antibody with yeast killer toxin for the binding site of MAb KT4. When purified by affinity chromatography, the anti-idiotypic antibodies exerted their killer activity in a CFU assay against the yeast killer toxin-susceptible yeast cells used for the challenge (Fig. 8). The candidacidal activity of the anti-idiotypic antibodies was totally neutralized by previous absorption with MAb KT4, thus attesting to the specificity of their action. These results established that immunity against infectious agents may be sustained by anti-idiotypic antibodies mimicking the receptor-mediated activity of antimicrobial compounds. The new concept of idiotypic vaccination substantially differs from anti-idiotypic vaccination, since immunizing antibodies are not aimed at mimicking the relevant microbial antigen as a vaccine but are elicited by a complementary idiotype to mirror, in vivo, a microbicidal toxin (300).

The principle of idiotypic vaccination has been extended to immunization against vaginal candidiasis because mucosal diseases, caused mainly by *C. albicans*, are important in both immunocompromised patients and normal individuals. Vaginal candidiasis is widespread and is often an intractable disease in women. Despite rapid progress in the characterization of immunodominant antigens in *C. albicans*, there is no conventional procedure of immunization to protect against candidiasis (402). The intravaginal administration of MAb KT4 as a vaccine to obtain secretory anti-idiotypic antibodies mimicking the killer activity of the *P. anomala* toxin on the vaginal mucosa resulted in effective protection in a rat model of vaginitis. There was a significant decrease in CFU in the vaccinated animals compared with unvaccinated or isotype-matched irrelevant monoclonal antibody-immunized controls (301). The immunoprotection that resulted was associated with increasing titers of vaginal yeast killer toxin-like anti-idiotypic antibodies, primarily of the secretory immunoglobulin A isotype. The antibodies, purified by affinity chromatography against MAb KT4, conferred passive immunoprotection on unvaccinated animals experimentally infected with an intravaginal challenge of *C. albicans* cells. The affinity chromatography-purified, secretory anti-idiotypic antibodies, moreover, had candidacidal activity, since they were able to kill in vitro the *C. albicans* cells used for experimental infections. This killing activity was completely neutralized by absorption with MAb KT4.

Immunization through monoclonal antibodies bearing receptor-like idiotypes may represent a new approach to vaccination since anti-idiotypic antibodies do not merely replace the immunogen, as in anti-idiotypic vaccination, but also act as antibiotics on a receptor of the infectious agent not involved in the vaccination process. Anti-idiotypic antibodies elicited in vivo against monoclonal antibodies that neutralize the receptor-mediated activity of antimicrobial products could represent a model for immunoprotection against microbial agents in general.

Although the role of the conventional immune response in the protection of the immunized mice remains to be clarified, the in vitro killing activity of the affinity chromatography-purified mouse anti-idiotypic antibodies on *C. albicans* cells implied an in vivo antibiotic activity.

The possibility exists that, in animals primed with MAb KT4, *C. albicans* cells represented a booster injection because of their yeast killer toxin cell wall receptors that were structurally similar to the monoclonal antibody idiotype responsible for immunization.

Natural Human Yeast Killer Toxin-Like Antibodies

Previous findings obtained from idiotypic vaccination suggested that anti-idiotypic antibodies represent the internal image of the yeast killer toxin. Thus, there would be a structural homology between the idiotype of MAb KT4 and the yeast killer toxin cell wall receptor of *C. albicans* cells. This assumption is corroborated by the competition of yeast killer toxin-like anti-idiotypic antibodies with the *P. anomala* killer toxin for the binding site of MAb KT4 and the specific reactivity of yeast killer toxin-like anti-idiotypic antibodies with *C. albicans* yeast killer toxin receptors (291, 295, 297, 300, 301). Theoretically, the production of yeast killer toxin-like antibodies should be

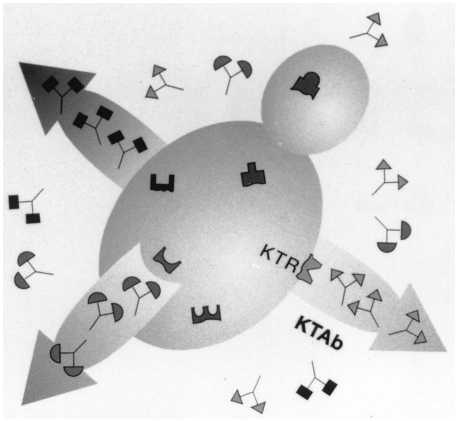

FIG. 9. Speculative representation of the production of candidacidal yeast killer toxin-like antibodies (KTAb) elicited by yeast killer toxin cell wall receptors (KTR) of *C. albicans.*

stimulated in unvaccinated animals immunized with killer toxin receptor-bearing *C. albicans* cells or recalled in those primed with MAb KT4 by a booster dose of the cells (Fig. 9). Accordingly, yeast killer toxin-like anti-receptor antibodies should also occur in the human antibody repertoire, particularly in those immunocompetent patients undergoing multiple infections or recurrent diseases as well as extensive colonization with *C. albicans*. Intravaginal or intragastric challenge of yeast killer toxin cell wall receptor-bearing susceptible *C. albicans* cells was shown to recall yeast killer toxin-like anti-idiotypic antibodies in the vaginal fluid of animals previously immunized by idiotypic intravaginal vaccination with MAb KT4. Yeast killer toxin-like anti-receptor antibodies were also elicited in animals that were repeatedly infected intravaginally with receptor-bearing yeast killer toxin-susceptible *C. albicans* cells but never immunized with MAb KT4. Anti-receptor antibodies, functionally mimicking the yeast killer toxin, were also consistently found in the vaginal fluid of women infected with *C. albicans* who obviously had never been exposed to MAb KT4 idiotypic vaccine. Natural human yeast killer toxin-like antibodies proved to be as candidacidal in vitro as those raised in rat vaginas by idiotypic vaccination. Their fungicidal activity was totally abolished by previous absorption with MAb KT4. Affinity chromatography-purified natural human yeast killer toxin-like vaginal antibodies conferred immunoprotection against experimental vaginal candidiasis when passively transferred to the vaginas of laboratory animals (302). Moreover, these antibodies detected the putative yeast killer toxin cell wall receptors on *C. albicans* germinating and budding cells by immunofluorescence (Fig. 10). Significantly, the location and distribution of the specific receptors corresponded to those observed by using yeast killer toxin-like rabbit anti-idiotypic antibodies obtained by idiotypic vaccination. Even though a clinical relationship between the occurrence of natural human killer toxin-like antibodies and the severity of infection has not been established, these findings would add new relevance to antibody-mediated immunoprotection against candidiasis, in contrast to cell-mediated immunity, whose importance has received a general consensus.

Perspectives

Despite the conceptual similarity of the steric interaction between yeast killer toxins and their specific receptors and antigen-antibody complementarity, it may seem implausible that antibody molecules share idiotypes with such unrelated structures. Although experimental findings have shown that

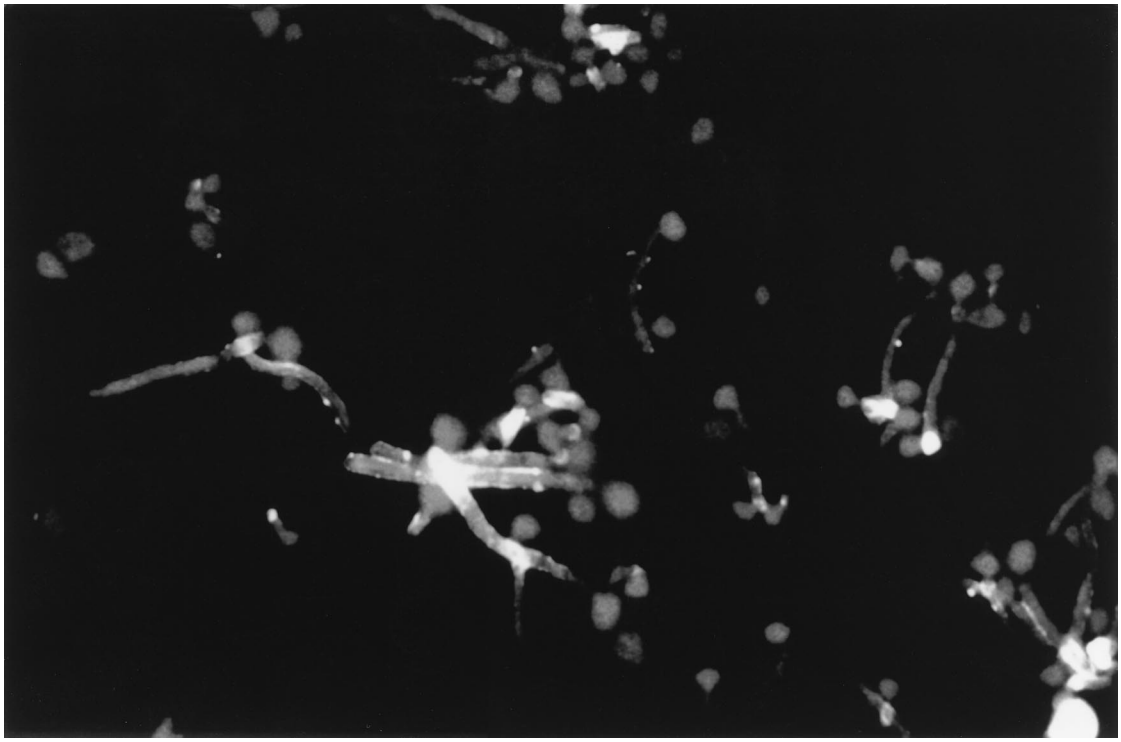

FIG. 10. Visualization of putative *P. anomala* killer toxin cell wall receptors in *C. albicans* by immunofluorescence with natural human candidacidal yeast killer toxin-like antibodies.

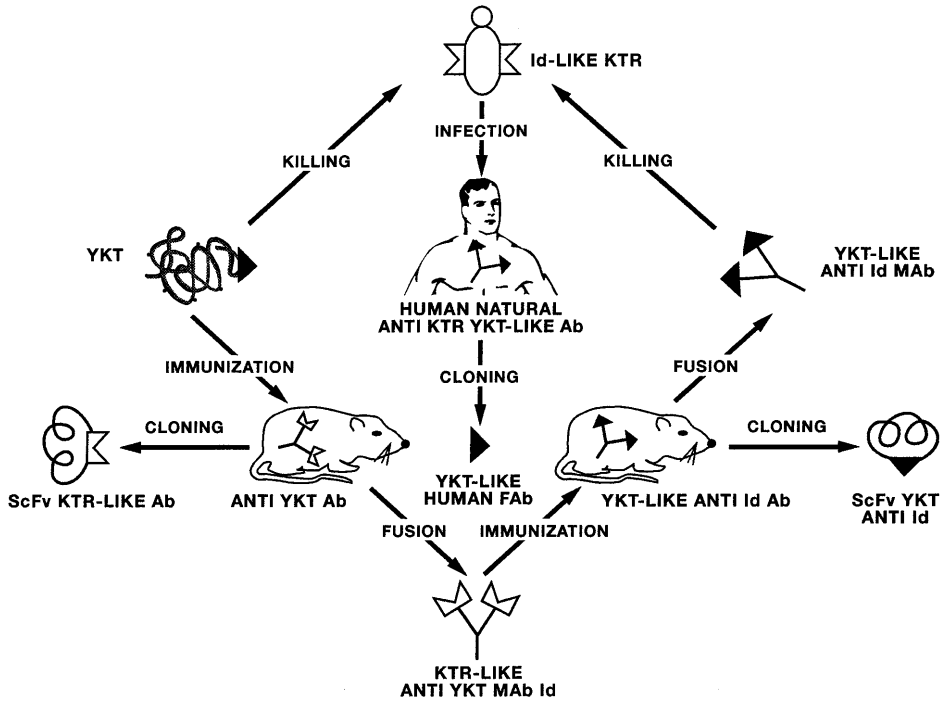

FIG. 11. Idiotypy of killer toxins, killer toxin receptors, and killer toxin-like and receptor-like antibodies. Abbreviations: YKT, *P. anomala* killer toxin; KTR, killer toxin receptor; Id, idiotype; AntiId, anti-idiotype; ScFv, single-chain fragment variable; Ab, antibody.

the amino acid sequence of the reovirus hemagglutinins shares a region of homology with the light chain of an internal image-bearing monoclonal anti-idiotype, further molecular biological studies will be required to explore any sequence homology among the genes encoding yeast killer toxin and anti-idiotypes as well as those encoding yeast cell wall receptors and idiotypes (40). Studies at the molecular level, however, suggest that the mirror configuration of anti-idiotypic antibodies with original antigens is functional rather than an exact topological reproduction and basically involves similar binding interactions. This assumption would seem plausible, particularly for antibodies mimicking antigens other than proteins (223).

Nevertheless, hybridoma technology (202) and molecular immunology (72) provide tools to synthesize antibody derivatives that could exert antimicrobial activity (Fig. 11). Monoclonal yeast killer toxin-like anti-idiotypic antibodies exerting a candidacidal activity and interacting with yeast killer toxin cell wall receptors of *C. albicans* have been produced by idiotypic vaccination with MAb KT4 (303). The genes encoding the variable regions of yeast killer toxin-like anti-idiotypic antibodies have been cloned in a phagemid library and expressed in the single-chain format (ScFv). ScFv yeast killer toxin-like anti-idiotypic antibodies proved to be candidacidal in vitro and to compete with killer toxin for the specific receptors on *C. albicans* cells (231). The fungicidal activity of monoclonal and ScFv yeast killer toxin-like anti-idiotypic antibodies was neutralized by MAb KT4. Notably, monoclonal and recombinant yeast killer toxin-like anti-idiotypic antibodies proved to exert a significant therapeutic activity when administered at the time of challenge (i.e., simultaneously with inoculation of fungal cells) or postchallenge (i.e., 1 to 5 days after the administration of fungal cells) in an experimental model of vaginal candidiasis (231). The variable regions of human fungicidal antibodies could be obtained by assembling combinatorial libraries (12) from selected donors undergoing natural infections with yeast killer toxin receptor-bearing pathogenic microorganisms. Recombinant fungicidal antibodies of human origin could represent a new therapeutic approach for the immunotherapy of systemic fungal diseases. These antibodies would be reproducible, have unlimited availability, and be free of undesired toxic effects. Many important questions still remain to be solved, such as the characterization, nature, and expression of the common transphyletic yeast killer toxin cell wall receptors in pathogenic microorganisms and the life span of their interaction with the host immune system. It is intriguing to speculate that the host immune system may exploit the yeast killer toxin receptor of infecting pathogens to produce microbicidal antibodies that mirror the competitive events occurring among microorganisms in natural habitats (63).

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