Killer of \textit{Saccharomyces cerevisiae}: a Double-Stranded Ribonucleic Acid Plasmid

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INTRODUCTION

Certain strains of the yeast \textit{Saccharomyces cerevisiae} (called killers) secrete a substance (the killer toxin) which is lethal to other strains of the same species (called sensitives) \((46)\). This is illustrated in Fig. 1, where a seeded lawn of a sensitive strain was streaked with a killer strain and a sensitive strain. The killer strain prevented growth of the lawn. All wild-type killer strains are resistant to the effects of the toxin(s) they produce \((46)\). Thus, this system is analogous to the colicin systems \((32)\) and the killers of \textit{Paramecium} \((67a)\).

Current evidence indicates that the yeasts secreting the toxin(s) are those carrying a double-stranded ribonucleic acid (dsRNA) species encapsulated in intracellular virus-like particles. The maintenance or replication of the killer plasmid (dsRNA in virus-like particles) requires at least 10 chromosomal genes \((mak)\), whereas expression of killing and resistance requires 3 other chromosomal genes \((kex\) and \(rex)\). Defective interfering plasmid mutants ("suppressives") and plasmid mutants dependent on chromosomal diploidy for expression and maintenance have been described.

This review will treat the genetics and biochemistry of the non-Mendelian genetic element(s) responsible for the "killer character" of yeast, the secretion and structure of the toxin, and the mechanism by which the toxin kills sensitive cells. We will consider this plasmid's relationship to other plasmids in the cell. Some host genes having a role in replicating or expressing the information in the dsRNA plasmid also have a role in exclusively host processes, e.g., mating, sporation, growth, or respiration. The genetics of this plasmid and its interactions with its host will be an especially favorable model for the class of dsRNA viruses and for general host-virus relationships in eukaryotes.

Notation

\textbf{Phenotype notation.} A strain's phenotype is designated \(K^+\), \(K^-\), or \(K^0\) for normal killing ability, weak killing ability, or no killing ability, respectively; its resistance to killing may be, similarly, \(R^+\), \(R^-\), or \(R^0\). \(K\) and \(R\) here correspond to \(T\) and \(I\) used by Vodkin et al. \((73)\).

There are at least two different killer systems in yeast that show no cross-immunity (see below). The first is widespread in laboratory strains and has been studied extensively, whereas the second has been found exclusively in wine yeast (50-53). When a distinction is necessary, the killer phenotype of laboratory strains will be denoted K₁ R₁ and that of killer wine yeast K₂ R₂. When no distinction is made, K₁ and R are intended.

The killer genotype is denoted here as follows. Chromosomal mutations are indicated in the usual way; e.g., a mak2-1 denotes a strain of a mating type carrying a recessive mutation in the mak2 gene. Note that mak genes are needed for maintenance or replication of the killer plasmid whereas kex and rex chromosomal genes are needed for expression of killing and resistance, respectively (74). The killer plasmid residing in a strain is indicated in square brackets, [ ]. Examples are: [KIL-o], a sensitive strain carrying no killer plasmid (or one which is phenotypically and genetically inapparent); [KIL-k], a normal killer plasmid; [KIL-n26], a neutral plasmid (67) which gives an otherwise normal strain, the K⁻ R⁺ phenotype; [KIL-s3], suppressive, or defective, interfering plasmid (66); [KIL-h] and [KIL-c], heat cured (75) and cycloheximide cured (25), respectively; and [KIL-d30], a diploid-dependent plasmid (76). The letters KIL indicate that one is referring to the killer genome. This is followed by a small letter corresponding to the nomenclature devised by Somers and Bevan (67) and Somers (66). Finally, a number denotes the mutant or isolate number. Subscripts, e.g., [KIL-k₁] or [KIL-k₂], will be used to differentiate killer plasmids, in this case the usual killer plasmid and that found in wine yeast, respectively. This nomenclature should be replaced by the usual gene designations as soon as a method is obtained for doing complementation tests and mapping with mutants of the plasmid. The killer plasmid notation used here follows the recommendation of Sherman and Lawrence (63).
Detection of the Killer Phenotype

The killer phenotype may be ascertained by the inhibition of growth of a lawn of a sensitive strain in a zone surrounding a killer strain (67). Several variables affect the sensitivity and specificity of this assay (83). The killer toxin is only active around pH 4.7 and is somewhat heat labile, so assays are run on buffered medium at a standard temperature (the author uses 20°C). Because the mating pheromone α-factor secreted by α cells inhibits the growth of a cells (23), we use a diploid sensitive strain as our indicator. All sensitive strains we have examined give roughly the same zone of inhibition with a given killer, but it is desirable to use a single standard sensitive strain to avoid variability from this source.

Measurement of resistance to the toxin is complicated in some strains by an inability to grow on the pH 4.7 medium. This is the case, for example, with K⁺ R⁻ strains.

GENETICS OF THE KILLER PLASMID OF S. CEREVISIAE

Yeast Genetics

Yeast is a simple eukaryote with a nuclear membrane, 17 chromosomes, mitochondria, endoplasmic reticulum, golgi bodies, and mitotic and meiotic division processes. The life cycle of S. cerevisiae (Fig. 2; reviewed in 57 and 63) involves stable haploid and diploid phases, during which each mitotic growth may occur. Haploid strains may be either a or α mating type, and strains of opposite mating types fuse with each other under growth conditions to yield diploid cells which are heterozygous (a/α) at the locus on chromosome III which determines the mating type. When such diploids are given acetate as the sole carbon source and starved for nitrogen, they undergo meiosis and encapsulate each of the four haploid meiotic products in a spore. The cell wall of the original diploid cell remains surrounding the four spores. Thus each "tetrad" of meiotic products is contained in an "ascus" (sac). The four spores of each tetrad are easily separated by micromanipulation and germinated, and the spore clones can be analyzed.

If the parents in a cross differ only in a single chromosomal gene, two of the spore clones of each tetrad will resemble one parent and the other two will resemble the other parent (2:2 segregation, e.g., mating type a or α in Fig. 2).

If the parents differ only in the presence or absence of a cytoplasmic genetic element (plasmid) such as mitochondrial deoxyribonucleic acid (DNA), then all four of the meiotic progeny in each tetrad will carry the plasmid and have the corresponding phenotype.

If the haploid parents carry differently marked plasmids of the same type, e.g., mitochondrial DNA carrying oligomycin resistance in one parent and carrying erythromycin resistance in the other parent, then segregation of the parental characters during the mitotic growth of the diploid zygote is generally seen. Chromosomal markers only rarely segregate during mitosis.

The life cycle of yeast makes it possible to isolate mutants in haploid strains, do complementation analysis using diploids, and study the segregation of markers in meiosis or mitosis.

Inheritance of the Killer Character in Wild-Type Strains

The killer character of S. cerevisiae is controlled, at least in part, by a nonchromosomal genetic element, as evidenced by the following. First, when wild-type killers (K⁺ R⁺) are mated with wild-type nonkillers (K⁻ R⁻), the diploids are all K⁺ R⁺, and all meiotic segregants are K⁺ R⁺ (67) (Fig. 3; Table 1). If the difference between killers and nonkillers were at a single chromosomal locus, 2 K⁺ R⁺:2 K⁻ R⁻ segregation would be expected. Some wild-type yeasts are K⁻ R⁺ (neutrels), neither secreting toxin nor being sensitive to it. When these are crossed with wild-type sensitive strains, a similar pattern of inheritance is seen, with all diploids being K⁺ R⁺, and all meiotic segregants K⁻ R⁻ (67) (Table 1). The killer phenotype of wine yeast mentioned above, K⁺ R⁺, shows the same 4 K⁺ R⁺:0 pattern of inheritance when mated with wild-type sensitive strains.
Second, when neutral (K⁻ R⁺) and killer (K⁺ R⁺) strains are mated, Bevan and Somers (7) found that a single diploid clone could give rise to mitotic segregants of both K⁺ R⁺ and K⁻ R⁻ phenotypes. Furthermore, when these killer diploids were sporulated, the meiotic segregants included both killer and neutral (K⁻ R⁺) spore clones. The killer spore clones, like the killer diploids from which they arose, also showed mitotic segregation of killer and neutral traits; i.e., when a killer spore clone was replicated for single colonies, some of these colonies were neutrals, whereas the rest were killers. This mitotic segregation again indicates a nonchromosomal mode of inheritance and suggests that the neutral strains carry a defective killer plasmid.

A third line of evidence for the nonchromosomal inheritance of the killer is the efficient "curing" of the killer trait by treatments not known to be mutagenic for chromosomal loci. When killer cells are plated for colonies in the presence of low concentrations of cycloheximide (0.5 μg/ml) (25), a majority of the clones are K⁻ R⁻. Growth of cells at elevated temperatures (37 to 40°C) similarly results in a high frequency of conversion of killers to K⁻ R⁻ (75). These cured strains resemble wild-type sensitives in all respects and upon crossing with other K⁻ R⁻ strains never produce killer diploids or killer meiotic progeny. In each case, the loss of killer phenotype is not due to the induction of a chromosomal defect, since crosses of heat- or cycloheximide-cured K⁻ R⁻ strains with wild-type killers yield consistent 4 K⁺ R⁺:0 segregation. Neutral strains may also be heat cured (75).

The mechanism of curing by heat or cycloheximide is not known, but cycloheximide also affects secretion of the killer toxin. Incubation of killer cells in 2 μg of cycloheximide per ml for 1 h reduces toxin secretion by 95 to 97% (13), thus suggesting that the killer toxin protein is made on cytoplasmic ribosomes. Other interpretations, however, are possible.

The killer (K₁ or K₂) and neutral traits can also be eliminated by acridine orange but not by acriflavin, proflavin, or ethidium bromide (53) (see below). In this study, only acridine orange failed to induce respiratory deficiency. Presumably, acridine orange is acting to eliminate the cytoplasmic killer genome, but this has not yet been verified.

The cytoplasmic killer genome of K₂ R⁺ killers is "suppressed" by killers or neutrals with respect to K₁ killing. Thus K₂ R⁺ crossed with wild-type sensitive strains (K⁻ R⁻) yield only K₂ R⁺ diploids and 4 K₂ R⁻:0 meiotic segregants. But when K₂ killers are crossed with K₁ killers, the diploids and meiotic segregants are all K₁. Likewise, crosses of K₂ with K⁻ R⁺ neutral segregants yield only K⁻ R⁺, neutral diploids, and meiotic segregants (52). This phenomenon is not understood.

**KILLER PLASMID MUTANTS**

Neutral Plasmid Mutants

Occasionally, wild-type strains have the K⁻ R⁻ phenotype and are called neutrals (67). As discussed above, these strains appear to have a plasmid which confers only resistance [KIL-n]. Neutral strains constitute a frequent class of mutant derived from killer strains and show the same pattern of inheritance as wild-type neutral strains. However, not all K⁺ R⁺ strains are of the neutral type. They may also be kex [KIL-k], sek [KIL-o], or [KIL-d] (Table 1), as we shall discuss below.

Neutrals for the K₁ killer (K⁻ R⁺) were isolated from homothallic wine yeasts by Naumov (49a). One of these strains resembled the neutrals of the K₁ killer in showing non-Mendelian inheritance and curability by acridine orange. Three others showed meiotic segregation indicative of chromosomal inheritance. Resistance of the chromosomal mutants was dominant to sensitivity, and resistance to killing was not eliminated by treatment with acridine orange. This last finding suggests that the resistance chromosomal gene is not one of the chromosomal genes needed to maintain the plasmid (see below). These mutants also differ from the
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* All the data shown here were obtained using the killer in the usual laboratory strains.

* Diploids formed in this cross are a mixture of K⁺ R⁺ and K⁻ R⁻ cells. On meiosis, these yield the indicated segregants (76). The K⁻ R⁻ diploids arise from haploids which have lost the d plasmid.

* M. Leibowitz and R. Wickner, unpublished manuscript; H. Bussey, personal communication.
 recessive K- resistant mutants (sek) described below, which were derived from haploid K- sensitive strains.

Suppressive Plasmid Mutants

Somers (66) described a class of mutants of a killer (or neutral) strain which had become K- R-, but, unlike wild-type nonkillers or heat- or cycloheximide-cured K- R- strains, these mutants were partly or entirely "dominant" to wild-type killers. These mutants are called "suppressive" sensitive in analogy with comparable mitochondrial mutants first described by Ephrussi et al. (24). The suppressive sensitive mutants of Somers, when mated with K+ R+ strains, yield diploids which, initially, are a mixture of K+ R+ and K- R- cells (66). On subcloning these diploids, the killer character is always eventually lost. These K- R- diploids yield 4 K- R-:0 segregation on meiosis. Moreover, each of these K- R- haploid segregants carries the suppressive trait; i.e., when it is mated with a killer, it yields K- R- diploids.

Thus the suppressive trait shows non-Mendelian segregation, suggesting that this is a mutation of the killer plasmid, denoted [KIL-s]. Indeed, as shall be discussed below, it appears to be a deletion of part of the killer plasmid. The physiological basis of the suppressive character is unclear, but it cannot be interference with expression of the killer character, since some of the diploids formed by mating [KIL-s] and [KIL-k] strains are transiently killers.

Further evidence that the suppressive [KIL-s] plasmid is, as this notation suggests, a mutant of the killer plasmid is that it is dependent on the chromosomal m gene (see below) for its maintenance, as is the wild-type killer plasmid [KIL-k] (66).

Diploid-Dependent Plasmid Mutants

Another class of mutants of the killer plasmid depends upon chromosomal diploidy for the expression of plasmid functions and for replication or maintenance of the plasmid itself (76; Table 1). These mutants are not defective in any chromosomal gene needed for expression or replication of the normal killer plasmid. Haploids carrying these mutant plasmids (called d for diploid-dependent) are either unable to kill or unable to resist being killed or both and show frequent loss of the plasmid. The wild-type phenotype (K+ R+) is restored by mating the d plasmid-carrying strain with either: (i) a wild-type sensitive strain which apparently has no killer plasmid; (ii) a strain which has been cured of the killer plasmid by growth at elevated temperature; (iii) a strain which has been cured of the plasmid by growth in the presence of cycloheximide; (iv) a strain which has lost the plasmid because it carries a mutation in a chromosomal mak gene (needed for plasmid replication, see below); or (v) a strain of the opposite mating type which carries the same d plasmid and has the same (or another) defective phenotype. This indicates that the restoration of the normal phenotype is not due to recombination between plasmid genomes or complementation of plasmid or chromosomal genes.

Sporulation of the phenotypically K+ R+ diploids formed in matings between d and wild-type nonkiller strains or other d strains yields tetrads, all four of whose haploid spores are defective for killing or resistance or maintenance of the plasmid or a combination of these. Every defective phenotype may be found among the segregants of a single diploid clone carrying a d plasmid. These defective segregants resume the normal killer phenotype in the diploids formed when a second round of mating is performed, and the segregants from a second round of meiosis and sporulation are again defective (76).

In contrast, crossing [KIL-d] haploids with [KIL-k] haploids yields only K+ R+ diploids, and these diploids yield only K+ R+ haploid meiotic segregants. Thus, the [KIL-d] plasmid is suppressed by the normal killer plasmid.

CHROMOSOMAL GENES INVOLVED IN KILLER PLASMID EXPRESSION AND REPLICATION

Chromosomal Killer Expression (kex) and Resistance Expression (rex) Genes

Three chromosomal genes that are essential for expression of plasmid information (74) have been identified. kex (for killer expression) strains were isolated as K- mutants of a killer strain and have the K- R+ phenotype. Unlike the plasmid mutants of the same phenotype called "neutrals," kex mutants yield K+ R+ diploids in crosses with wild-type nonkillers [KIL-o] or killers [KIL-k]. Sporulation of such diploids yields 2 K+ R+ :2 K- R- segregation (Table 1). The K+ R+ segregants from kex X [KIL-o] behave as normal killers. This indicates that kex strains carry a normal killer plasmid. If the killer plasmid is eliminated from kex strains by heat or cycloheximide curing, the strains become R-. When such a kex- K- R- strain is then crossed with a normal killer, 2 K- R+ :2 K+ R+ segregation is again seen, indicating that the normal killer plasmid requires the kex genes for expression of killing (7b).

Two kex genes have been defined by comple-
mentation and allele tests of 28 independent mutant isolates. kex1 is located on the left arm of chromosome VII, whereas kex2 is on chromosome XIV (77b; Fig. 4).

The rexl gene is a chromosomal gene needed for expression of resistance to killing but not for plasmid maintenance or for killing expression (74). Such strains are very unstable because of
their suicidal tendencies. However, they can be stored either as heterozygous diploids or as K-R haploids which have been cured of the killer plasmid (M. Leibowitz, unpublished observations).

**Mating and Sporulation Defects of *kex2* Mutants**

In the course of genetic analysis of *kex* mutants, it was noted that all *kex2* mutants showed defects in the sexual cycle (42, 43). *kex2* strains of α mating type show a marked defect in mating ability, whereas a *kex2* and all *kex1* strains mate normally. In the process of mating, each parent secretes pheromones which produce G₁ arrest and cell elongation in the other parent (33, 45, 64; Fig. 5). The polypeptide α-factor secreted by normal α cells has both of these effects on normal α cells (23). α *kex2* cells fail to secrete α-factor, but supplying α-factor to them does not correct their mating defect (42, 43). Thus their defect is more complex. Normal α cells secrete two pheromones, α-factor I which arrests α cells at G₁, (80) and α-factor II which produces elongation of α cells (V. MacKay, personal communication). α *kex2* cells respond to α-factor I but not to α-factor II. *kex2* strains of mating type a mate, secrete α-factor I, and respond to α-factor normally (43).

Diploids that are homozygous for the *kex2* mutation, unlike wild-type or heterozygous diploids, fail to undergo meiosis and sporulation, with the defect occurring late in the meiotic cycle after recombination and DNA synthesis have occurred. Diploids homozygous for *kex1* show normal meiosis and sporulation. These same defects in the sexual cycle are present in *kex2* mutants independent of the presence or absence of the killer plasmid (43). Thus, the *kex2* gene, which maps at a site distinct from the mating type locus, is required for toxin production in killer plasmid-carrying strains and for normal α-specific mating function and meiosis in all strains.

The primary defect in *kex2* strains is unknown. It cannot be a general secretion defect, since at least a-factor I is made and secreted by a *kex2* strains and supplying α *kex2* strains with α-factor and any other possible diffusible mating factors does not restore the mating defect.

**Chromosomal Genes Essential for Plasmid Maintenance or Replication**

In analyzing sensitive strains in their laboratory stocks, Somers and Bevan (67) found that some carried a chromosomal allele, m, which made them unable to kill or resist killing. On crossing *m* K⁻ R⁻ strains with K⁺ R⁺ strains, K⁻ R⁺ diploids were formed which yielded 2 K⁻ R⁻:2 K⁺ R⁺ segregation on meiosis (see Table 1). Killing ability could not be recovered from the K⁻ R⁻ segregants by mating and sporulation, unlike the *kex* mutants described above, where normal killer strains could be obtained by mating with wild-type [KIL-o] strains. The neutral plasmid (see section III) also needs the product of the *m* gene as does the killer plasmid [KIL-k] (50).

To show that *m* strains were defective in plasmid replication or maintenance, *m* spores from an *m/+* K⁺ R⁺ diploid were germinated and allowed to grow for only a few generations. These microcolonies were then mated with wild-type nonkillers. About half of the diploids formed were killers of the genotype *m/+* [KIL-k]. Thus the *m* gene does not prevent a spore from getting the killer plasmid, but makes it unable to maintain it (67).

Many other chromosomal genes are essential for maintenance of the killer plasmid (Fig. 4). These include *pets* on chromosome III (8, 25), *mak1* and *mak8* on chromosome XV (74, 77), *mak3* and *mak6* on chromosome XVI, *mak4* and *mak5* on chromosome II, and *mak7* on chromosome VIII (77). *mak2* (74) has not been mapped. The *m* gene is located on chromosome V (Bevan and Theivendirarajah, personal communication; 77).

One allele of *mak1* results in temperature sensitivity for growth (77). Spontaneous *pets*
mutants have three defects: they cannot maintain the killer plasmid, they are respiratory deficient (8, 25), and they are temperature sensitive for growth (77). The respiratory deficiency of *pets* strains is due to the complete loss of mitochondrial DNA as judged by CsCl density gradient centrifugation of DNA labeled in vivo (M. Leibowitz, unpublished observations). Thus, *pets* is needed to replicate or maintain two distinct nonchromosomal genomes. The temperature sensitivity for growth of *pets* strains has not yet been examined in detail.

In studying *mak* mutants, we have encountered several cases in which killing ability appeared to be independent of one or more *mak* gene products (77a; R. Wickner and M. Leibowitz, manuscript in preparation). In crosses of the type *mak K·R·* × wild-type *K+ R+*, killer sectors have been observed in spore clones that were otherwise *K− R−*. Isolates from many such sectors are mitotically stable killers carrying both the original *mak* mutation and a distant dominant centromere-associated change that makes the strain *K+ R+* in spite of carrying the original *mak* mutation. They are not simply translational suppressors. These dominant centromere-associated changes are unstable to heat or cycloheximide, the same treatments that cure the normal killer plasmid. The nature of the *mak*-bypass change in these strains is not yet clear.

Woods et al. (84) have reported a spontaneous *K· R·* mutant derived from a sensitive strain. The small amount of killer activity of this strain showed greater stability to heat or pH 5.8 than the usual killer toxin. The *K· R·* phenotype (called killer/sensitive by the authors, although they report that weak resistance to the usual killer toxin is present in the mutant) is recessive in a cross with an *m* [KIL-ol] strain (diploids all *K− R−*) and reappears among the meiotic progeny. Unfortunately, a cross with an *m* [KIL-ol] is not reported, so it is not clear whether this trait is chromosomal or cytoplasmic. When these *K· R·* strains are crossed with normal killer or neutral strains, the diploids and all meiotic progeny show the normal killer or neutral phenotype. No segregants were obtained with the killer/sensitive phenotype. It would be of interest to know whether [KIL-k] or [KIL-n] strains are killed by the Woods strain. If the toxin of this strain had the same spectrum of action as the normal killer toxin, the origin of this mutant from a sensitive strain would suggest that the killer toxin was coded by a chromosomal gene and not by the killer plasmid. The absence of the killer dsRNA in the strain of Woods et al. (84; see below) is consistent with this notion. However, no clear conclusions can be reached without further studies of this mutant, and the relationship between this strain's toxin and the usual toxin remains unknown. Attempts in our laboratory to isolate killers from wild-type *K− R−* strains or from *sek−* strains (see section VI) have been unsuccessful (J. Marans, M. Leibowitz, and R. Wickner, unpublished observations).

**EVIDENCE THAT THE KILLER PLASMID IS A dsRNA SPECIES IN VIRUS-LIKE PARTICLES**

dsRNA in Killer Yeast

Killer yeasts contain three species of dsRNA, called M (or P2), L (or P1), and XL, with molecular weights variously estimated at 1.4 × 10^6 to 1.7 × 10^6, 2.5 × 10^6 to 3.0 × 10^6, and 3.8 × 10^6, respectively (6, 73, 77). Only the presence of the M species has been correlated with the presence of the killer plasmid (6, 73, 77, 77b) as will be detailed below (see Table 2). The M species is the least abundant, comprising only about 10% of total dsRNA. There are about 100 copies of L dsRNA and 12 copies of M dsRNA per haploid cell (73, 77), assuming there are 50 μg of total RNA per 10^9 cells (8a).

Berry and Bevan (5) first reported an RNA species in some killer strains which was partly resistant to ribonuclease (RNase) in 0.15 M NaCl. However, this species was not correlated with the killer character nor was it well characterized as dsRNA. Vodkin and Fink (72) isolated a species of RNA from a particulate fraction of killer yeast that was clearly shown to be double stranded by its sensitivity to RNase III (60), by its resistance to pancreatic RNase in 0.3 M NaCl, and by its behavior on cellulose column chromatography (26). They found various dsRNA's in killer and nonkiller strains, but the species discussed above was found in the particulate fraction only in killers. In their later work, these authors state that this species was the "L" species of dsRNA whose presence does not appear to be closely related to the killer character. Moreover, others (1, 9, 35) (see below) have found L dsRNA in virus-like particles in nonkiller strains.

Bevan et al. (6) isolated dsRNA from a phenol-detergent extract by cellulose chromatography. This material from killer strains contained two species of dsRNA, the larger (P1 or L) of molecular weight 2.5 × 10^6 and the smaller (P2 or M) of molecular weight 1.4 × 10^6, using *Aspergillus foetidus* virus dsRNA's (59) as standards. The L species melted with sharp hyperchromicity at 99.3°C in 0.15 M NaCl at pH
7.0. It contained 45% guanine (G) plus cytosine (C) with G = C and A = U and induced interferon production in mouse L cells consistent with a double-stranded structure. The L species was present in larger amounts than M, comprising 70 to 90% of the total.

M was shown to be dsRNA by its behavior on cellulose columns and its pancreatic RNase resistance. Vodkin et al. (73), using a different isolation procedure, found both L and M to be RNase III sensitive. In wild-type killers, 0.1% of total cellular RNA was recovered in L dsRNA and 0.006% in M dsRNA (73), but the efficiency of the initial extraction of RNA from cells was not reported. Wickner and Leibowitz (77a, 77b) efficiently extracted total nucleic acids, selected the dsRNA fraction by cellulose chromatography, and analyzed this material on gels. They confirmed the results of Vodkin et al., recovering 0.1% of total nucleic acids as dsRNA (mostly L), and of this 7% was in the M species. One can calculate the number of L and M molecules per cell from this information and the total cellular RNA content (50 μg per 10⁶ haploid cells [8a]). This gives values of about 100 L molecules and 12 M molecules/cell. Vodkin et al. also noted an excess of A residues in the M species and in the S species found in suppressive strains (see below). Shalitin and Fischer (62) have found that 50% of the dsRNA isolated by electrophoresis of RNA from killer yeast was bound to poly(U)-Sepharose, suggesting that the L and M species may have poly(A) sequences that are available for hybridization.

A third species of dsRNA, called XL, has recently been found in all strains examined, including killers, wild-type nonkillers, and various mutants (77, 77b). This species has a molecular weight estimated at 3.8 x 10⁶ by comparison with φ6 dsRNA segments, whereas the same comparison yields estimates of 3.0 and 1.7 x 10⁶ for L and M, respectively. XL is double stranded, based on its behavior on cellulose columns and its pattern of resistance to pancreatic RNase in low and high salt (77). It is intermediate in abundance between the L and M species. XL seems to be a discrete species, and not a gel artifact, but may be related in some way to L. For example, XL might represent replicating L molecules or L molecules in some particular conformation.

Bevan et al. (6) found a correlation between the presence of full-size M and the killer character. All killers and neutrals carried both L and M, whereas all 23 sensitive lacked M. Three sensitive did have a second band of dsRNA, which was reduced in molecular weight compared to the M species in normal killers. Strains mutant in the chromosomal m gene needed for plasmid maintenance or replication invariably lacked M and often lacked L as well. As no strains carrying M alone were reported, nor have any been subsequently found, the role, if any, of L in the killer phenomenon remains in doubt.

Further correlation of the M species with the cytoplasmic killer genome was made by Vodkin et al. (73). Spontaneous nonkiller derivatives of killer strains lacked M and displayed a decrease or absence of L. In contrast, cyclohexi-
mide-cured strains that also lack M have an increase in L.

Perhaps most important was the finding by Vodkin et al. (73) and Teen et al. (71) that strains carrying a suppressive plasmid (two independent isolates) no longer had a species the size of M but had a smaller species of about $5 \times 10^4$ molecular weight. The inference that this species (S) is a deletion mutant of the M species has since been confirmed (H. Fried and G. Fink, personal communication). A strain lacking resistance (K$^+$ R$^-$), but showing a cytoplasmic pattern of inheritance unlike rex1, was examined and had normal amounts of L and M but in addition carried a small species of molecular weight $2.5 \times 10^4$. A superkiller strain (detected by an increased zone of killing in the usual plate test) had 2.5-fold more M dsRNA than the wild-type, whereas m and pets strains lacked M entirely (72). These correlations provide rather strong circumstantial evidence that the M dsRNA species is the cytoplasmic killer genome.

Examination of kez1 and kez2 strains showed that they have XL, L, and M species (77b), as expected from their genetic properties: crosses of kez1 or kez2 strains with [KIL-o] strains yield segregants, half of which are K$^+$ R$^+$ [KIL-k]. mak1 through mak8 were also examined, and each strain contained normal amounts of XL and L but lacked the M species. Plasmid diploid-dependent mutants had normal amounts of all three species (76). These findings further strengthen the notion that M is the killer genome.

Shalitin and Fischer (62) argue that the M and L species are encoded by nuclear DNA. Their evidence is that poly(A)-containing RNA, purified by poly(U)-Sepharose, from two killer strains shows a 1.7-fold higher rate of annealing (C$_{50\%}$) than RNA purified from two unrelated nonkiller strains. They show that 50% of killer RNA binds to poly(U)-Sepharose and speculate that all poly(A) RNA is killer RNA or killer RNA precursor. From this notion and the extent of hybridization of poly(A) RNA with DNA, they argue that there are $\sim35$ killer cistrons/haploid nuclear genome. However, these conclusions are open to criticism. The hybridization studies were carried out with the total RNA isolated by poly(U)-Sepharose from each strain. These workers did not hybridize killer RNA with cellular DNA, an experiment which would provide a definitive test of their hypothesis. Furthermore, Sripathi and Warner (personal communication) have found that 80 to 85% of polysomal RNA (mRNA) labeled in a 5-min pulse binds to poly(dT)-cellulose. It seems unlikely that killer mRNA constitutes 80 to 85% of total message, since the killer-associated M species constitutes only 0.01% of the total cellular RNA. The hybridization experiments of Shalitin and Fischer (62) are also inadequate, because the RNA and DNA concentrations used were so low that estimates of the complexity of the RNA and its representation in the DNA are not possible. Finally, the relatively small difference in hybridization with DNA from a killer strain between the RNA from killer (A364A and a relative) and nonkiller (isogenic with S288C) strains might have been due to some other difference between these genetically distinct strains.

**Virus-Like Particles Contain the L and M dsRNA**

Circular particles 33 to 35 nm in diameter (160S) containing daRNA of molecular weight $2.5 \times 10^4$ (like P1 or L) were first isolated by Buck et al. (9) from several sensitive strains of yeast. Killer strains also had such virus-like particles and both P1 (L) and P2 (M) daRNA could be extracted from rapidly-sedimenting material containing such particles in proportions similar to those present in whole nucleic acid extracts (35). M dsRNA-containing particles sedimented more slowly than L-containing particles and could be obtained free of L, suggesting that L and M are associated with separate particles. Adler et al. (1) found RNA-containing particles 40 nm in diameter in killer, neutral, and sensitive strains, as well as "empty" particles. The RNA-containing particles had a density of 1.40 g/cm$^3$ in CsCl and carried daRNA of molecular weight $2.54 \times 10^4$. Killer strains had, in addition, daRNA of molecular weights of $1.19 \times 10^4$ and $1.29 \times 10^4$ in the particles, but these seemed to be lost from particles on CsCl density gradient centrifugation.

Thus the killer character of yeast appears to be due to the presence of virus-like particles carrying a daRNA genome. Viruses with daRNA genomes are widespread in nature (see reviews in references 38, 44, 44a, 81). They infect most vertebrates (including man), plants, insects, fungi, and bacteria, and it seems likely that study of the killer of yeast may shed some light on these other systems.

**THE KILLER TOXIN AND ITS MECHANISM OF ACTION**

Cell-free filtrates from cultures of killer cells can kill sensitive strains, but the killing substance does not multiply in sensitive strains;
i.e., it does not form plaques. Killer toxin is a protease-sensitive macromolecule secreted into the medium by killer strains (83). It is stable only in a narrow pH range, between 4.6 and 4.8, and is easily inactivated by temperatures above 25°C or by aeration in liquid medium. It is far more stable in agar or in liquid medium with gelatin. The toxin may be easily assayed by placing dilutions in wells in agar seeded with a sensitive strain and observing the size of the resulting zone of growth inhibition. The square of the diameter of this zone is proportional to the log of the killer toxin concentration (83).

Toxin production can also be measured crudely by replica plating colonies or patches onto a newly seeded lawn of a sensitive strain and observing a zone of growth inhibition as in Fig. 1.

Partially purified preparations of the toxin were first obtained by Woods and Bevan (83) using co-precipitation with ammonium sulfate of the toxin with gelatin from the medium. More recently, Palfree and Bussey (personal communication) have purified the killer toxin about 140-fold over the crude concentrated medium. They find that their purified preparations are about 90% carbohydrate (all D-mannose) and about 10% protein. A protein moiety (molecular weight of about 10,000) can be dissociated from the carbohydrate portion by detergent and seems to be the toxin-specific component, since nonkillers produce material similar to the carbohydrate-rich portion lacking this protein. Nucleic acids have not been detected in toxin preparations.

Exposure of sensitive cells to the killer toxin results in a delayed and roughly coordinate inhibition of DNA, RNA, protein, and polysaccharide biosynthesis and a turbidity change at a time when leakage of cellular adenosine 5'-triphosphate (ATP) into the medium begins (10-12). This leakage of ATP into the medium begins at about 60 min after exposure of cells to the killer toxin and is at least partly specific in that relatively little leakage of glucose, leucine, or macromolecules is observed, whereas complete depletion of intracellular ATP is seen. This is accompanied, however, by continued synthesis of ATP so that an amount of ATP accumulates in the medium equivalent to five times the original cellular ATP content (12). Whereas these facts point to the membrane as a site of action of the killer toxin, the long delay before these effects are seen suggests that the membrane may not be the initial site of action of the toxin.

In an attempt to dissect the process of killing, Bussey (10) has shown that over half of the cells which have adsorbed the toxin and would otherwise have died may be rescued by treatment with gluceses (a crude snail preparation which digests yeast cell walls). But the cell wall is not necessary for activity of the killer toxin. Spheroplasts of sensitive cells show a cutoff of [14C]glucose incorporation about 90 min after treatment with killer toxin, whereas spheroplasts of killer cells are unaffected (13).

Both killer and sensitive cells remove the toxin from solution. That this binding of the toxin (rather than nonspecific inactivation) is related to the action of the killer is suggested by the isolation of toxin-resistant mutants of sensitive cells which now no longer bind the toxin. Spheroplasts of such cells are still sensitive to the toxin. These results suggest that the toxin’s binding to the cell wall is part of its process of entry into the cell, but that the resistance of normal killer strains is not at this level (13).

There are at least three chromosomal cistrons, called sek (for sensitivity to killer) or kre (for killer resistance), recessive mutations which can result in conversion of a K- R+ strain to the K- R+ phenotype (13; M. Leibowitz and R. Wickner, unpublished; H. Bussey, personal communication).

**RELATION OF THE KILLER PLASMID TO OTHER NONCHROMOSOMAL GENETIC ELEMENTS**

Several traits have been described in yeast which, like the killer character, display non-Mendelian patterns of heredity. None of these shows any relation to the killer. Rho, the mitochondrial genome, has been identified with mitochondrial DNA and carries genes for mitochondrial ribosomal RNA, transfer RNA, and sites responsible for sensitivity to several antibiotics affecting mitochondrial protein synthesis or oxidative phosphorylation (for a recent review see 27). Ethidium bromide treatment of grande (respiratory competent, 𝜌+) cells results in total loss of mitochondrial DNA (29) and total conversion of cells to the petite (respiratory incompetent) phenotype due to loss of the cytoplasmic mitochondrial genome (65). Ethidium bromide treatment of killer grande cells sufficient to convert all the cells to petites does not produce any nonkillers (8, 25). In fact, after an ethidium bromide treatment producing more than 94% elimination of mitochondrial DNA, more than 99% of cells remained K- R+ (3). Furthermore, a majority of cells cured of the killer plasmid by heat or cycloheximide remain 𝜌+. Petites may also be induced with cycloheximide (25) or elevated temperatures...
dsRNA PLASMID: S. CEREVISIAE KILLER

DISTRIBUTION OF KILLER PHENOMENA AMONG FUNGI

The killer phenomenon was first reported among fungi in *S. cerevisiae* (46) and was shown to be widespread among laboratory strains of this species (25). It has now been observed in some brewing yeast (47), wine-making yeasts (50, 52), a baker’s yeast (56), and yeast containing saké (37). A large collection of yeast from many genera has been surveyed (56) for their ability to kill a particular sensitive strain of *S. cerevisiae*. Killer strains were found in the genera Debaromyces, Hansenula, Kluyveromyces, Pichia, Saccharomyces, Candida, and Torulasp. Based on the pH dependency of their activity, these killers could be assigned to four groups.

Killing by some *Ustilago maydis* strains of other strains of the same species, as well as other *Ustilago* species, has also been described (21, 31, 39–39b, 57, 82).

The killer of *S. cerevisiae* laboratory strains does not kill *Ustilago maydis* killer or sensitive strains, and *U. maydis* killers do not kill yeast (39). The *S. cerevisiae* toxin is lethal to *Torulasp glabra*, but not to *Candida albicans*, *Cryptococcus neoformans*, or *Schizosaccharomyces pombe* (15). *T. glabra* also produces a toxin that is lethal to killer and sensitive *S. cerevisiae* (14).

The killer found in the wine yeast *S. cerevisiae* M-437 (52) has a different specificity than most laboratory strains in that it is resistant to its own toxin but not to the common killer toxin and vice versa.

CONCLUSIONS

The killer character of yeast is a non-Mendelian trait which has been correlated with the presence of a dsRNA species of molecular weight about $1.4 \times 10^6$ to $1.7 \times 10^6$ encapsulated in isometric virus-like particles of about 40-nm diameter. The fact that this RNA species is double-stranded and is encapsulated suggests that it is indeed the cytoplasmic killer genome rather than a transcript of a DNA genome, but this conclusion will not be established until hybridization experiments show whether or not a DNA genome is present in killer strains. Infection of cells with the isolated virus-like particles or transformation with the isolated dsRNA would be convincing positive evidence of the nature of the cytoplasmic killer genome.

There is at present no information concerning how the killer dsRNA replicates; the process might resemble that described for reovirus (reviewed by Joklik [38]), but there are other
possibilities, including DNA involvement, as in RNA tumor viruses (reviewed by Temin (70)).

It is reasonable to expect that such a small genome, able to code for only about 100,000 daltons of protein would depend heavily on host proteins for its replication and for expression of its information. This expectation has been realized in the isolation of many mutants in chromosomal genes on which the plasmid depends. How these genes function in plasmid replication and expression is not known. Hopefully, in vitro complementation assays for mak proteins such as those used in bacterial and phage replication (4, 36, 54, 69, 78, 79) might allow purification and study of these proteins. Their role in host functions has also been suggested in several cases (pets, mak1, kex2) by the presence of other defects not related to the killer character in strains carrying mutations in these genes.

Although the cytoplasmic killer genome is necessary for toxin production and imparts resistance to a normal host, it is not yet clear that the toxin is coded for by the cytoplasmic genome. Proof of this normally requires, for example, the isolation of mutants that are temperature sensitive for killing ability and the demonstration that they secrete a thermostable toxin. If these mutants show cytoplasmic inheritance, the conclusion would be established. But since the isolated toxin appears to be mostly carbohydrate, it could be argued that a modification of the carbohydrate portion might affect the toxin's properties. This problem thus will require detailed studies. It is also possible that one of the kex genes codes for the toxin.

The relationship of the L and XL dsRNA species to the killer phenomenon is unclear. L and XL are maintained and replicated normally in mak1 through mak8 and pets strains so they certainly differ in their mode of replication and maintenance from M.

LITERATURE CITED


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