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Curing of a Killer Factor in *Saccharomyces cerevisiae*

(yeast/low pH/cycloheximide/ethidium bromide/"non-Mendelian")

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ABSTRACT **Many standard laboratory stocks of yeast are able to kill other yeast strains. This property has not been generally recognized because killing is observed only at low pH and not at the pH of standard media. In all strains examined, the genetic determinant for the killer trait shows non-Mendelian inheritance. The segregation patterns of our killer strains indicate that this killer determinant may be different from the killer previously described. Treatment of a killer strain with cycloheximide, but not with ethidium bromide, converts it into a sensitive nonkiller.**

Killer strains of yeast were first described by Bevan and Makower (1). Studies on the inheritance of the killer factor (2, 3) indicated that the killer was under the control of a cytoplasmic determinant (*k*), which required the presence of the nuclear gene *M* for maintenance in the cell. In the presence of the alternate recessive allele *m*, the killer factor failed to replicate and was diluted out. In many ways the formal genetic analysis of killer resembles that of kappa in Paramecium (4).

Studies on the structure and mode of action of the toxin produced by killer strains have indicated that it is a macromolecule excreted into the medium (5, 6). The killer substance is excluded from Sepharose 4B in several peaks (one with an apparent molecular weight in excess of two million), is sensitive to proteolytic enzymes and detergent, and appears to adsorb to yeast cell walls (6).

Our investigations show that yeast strains used in many laboratories are killers. The segregation pattern of this killer determinant is similar to, but not identical with, that described by Bevan. The killer determinant can be selectively removed by cycloheximide.

METHODS

Strains. All strains used in this analysis are listed in Table 1. The killer trait was observed when strain A8209B was replicated onto sensitive strain SCF1717. The strain containing *m*(o) was kindly sent by H. Bussey and J. Somers, and is the same as that described by Somers and Bevan (2).

Media. Standard media have been described (7). YPG is 1% yeast extract–2% peptone–3% glycerol. The ρ^- strains do not grow on YPG medium. Two low-pH media were used to test killing since the killer toxin is stable between pH 4.6 and 4.8 (2). A phosphate–citrate buffer, made by adjusting the pH of citric acid (final concentration 1 M) to 4.5 with K_2HPO_4, was used to lower the pH of the media to about 4.7.

Buffered methylene blue medium. After sterilization of YEPD medium in an autoclave, 100 ml of the phosphate–citrate buffer was added to 900 ml of medium. Methylene blue, a stain for dead yeast cells (8), is then incorporated into the medium at a final concentration of 0.003%.

Buffered YEPD. Standard YEPD medium (900 ml) is buffered with 100 ml of phosphate–citrate buffer added after sterilization.

Chemicals. Ethidium bromide and cycloheximide were purchased from *Calbiochem.*

Genetic Crosses and Ascus Dissection. Genetic crosses were performed as described (7). Diploids were obtained by selection, where both haploids had different auxotrophic requirements, or by micromanipulation of zygotes.

Detection of the Killer Trait. Strains growing on a YEPD plate are transferred by replica plating to low-pH plates that had previously been spread with a culture of sensitive cells. After incubation for two days at 22°, the killer colonies are surrounded by a clear zone on buffered YEPD medium. A clear zone fringed with a deep blue color indicated death of the sensitive cells on buffered methylene blue medium. Best results are obtained when about 5×10^5 sensitive cells are used for a lawn.

TABLE 1. *Strains used in this study*

Strain designation	Genotype
A8205B	*α his4-864 trp1 M*(k)
A8207B	*α his4-864 M*(k)
A8209B	*a his4-864 M*(k)
SCF1717	*α his1-123 M*(o)
A5164A	*a leu2-1 M*(o)
A5107B	*α leu2-1 M*(o)
A9193A	*a pets ade2 M*(o)
A9545D	*a ade2 m*(o)
S288C*	*α M*(o)
XT300-3A*	*α ade2-1 M*(o)
A664a/18A†	*α ura3 M*(o)
S18‡	*α ade2 m*(o)
Σ1278b§	*α M*(k)
D587-2A¶	*a M*(k)
A364A‖	*a ade1 ade2 ure1 tyr1 his7 lys2 M*(k)

* From Dr. R. Mortimer, University of California, Berkeley.
† From Dr. J. Marmur, Albert Einstein Medical School.
‡ From Dr. H. Bussey, McGill University.
§ From Dr. M. Grenson, University of Brussels, Belgium.
¶ From Dr. F. Sherman, University of Rochester (N.Y.).
‖ From Dr. L. Hartwell, University of Washington.

Abbreviation: YEPD medium, 1% yeast extract–2% peptone–2% dextrose.

Analysis of killer crosses can be confused by the mating type inhibition. When an *a* strain is used as the sensitive lawn on buffered YEPD plates, a zone of clearing is produced not only by the killer genotype *M*(k), but also by genotypes *M*(o) and *m*(o) of mating type α. On the buffered methylene blue plates the absence of a blue fringe around clear zones produced by the *M*(o) and *m*(o) strains shows that inhibition of growth, rather than killing, has occurred. This inhibition is no doubt a result of excretion of the α substance (9, 10), a small polypeptide under the control of mating type, which is thought to inhibit DNA synthesis in *a* strains. To avoid confusion in scoring meiotic progeny for the killer trait, an α-sensitive lawn was used. To test the sensitivity of strains an *a* killer was used. When the test is designed this way, the analysis of strains for presence of the killer and its absence (sensitivity) is unambiguous.

Standard definitions

A killer strain *M*(k) is defined by several properties:

Killing. Strains of genotype *M*(k) kill strains of genotype *M*(o) or *m*(o), the latter two being sensitives.

Immunity. Strains of genotype *M*(k) are not killed by strains of genotype *M*(k) in the standard petri plate test.

Non-Mendelian Segregation. In a cross of *M*(k) x *M*(o) all four spores are killers.

RESULTS

Meiotic segregation of the killer trait

The genetics of the killer trait in our strains appears to be formally analogous to the one found and described by Somers and Bevan (2). The results of crosses with several different strains (Table 2) indicate that the presence of a nuclear maintenance gene (*M*) is necessary for replication of the killer factor. If *M* is homozygous, as in a cross of *M*(k) x *M*(o), the diploid is a killer and all four spores have the genotype *M*(k). In M/m heterozygotes, e.g. a cross of *M*(k) x *m*(o), the diploid is a killer, and two of the spores are *M*(*k*) and two are usually *m*(o). The killer is extremely stable through meiosis in *M*(k) x *M*(o) crosses. In general, the presence of auxotrophic requirements does not influence the transmission of the trait. In crosses *M*(k) x *M*(o) where one of the strains contained the *ura3* marker, however, about 10% of the *ura3* spores had lost the killer trait.

A difference between the killers described previously and ours is evident in the cross of *M*(k) x *m*(o), as shown in Table 2. In three tetrads the *m* spores retained the killer trait during vegetative growth. The killer was unstable (in the presence of *m*), but continued subcloning of the haploid cells from one of these strains yielded *m* cultures in which 80–90% of the cells were killers. This aberrant behavior does not result from gene conversion of *m* to *M*. Subsequent crosses indicate that these unstable killer strains do not contain the *M* allele. In 112 tetrads from crosses of *m*(k) x *M*(o), the segregation of the killer trait is 2K:2NK in 99 of the asci and aberrant in 13 asci (3K:1NK or 1K:3NK).

Bevan and Somers (personal communication) have described a mutation *pets* that suppresses the propagation of the killer trait, maps close to the mating-type locus, and prevents growth on glycerol. We have found a spontaneously arising mutation with identical properties to the Bevan mutant. In crosses to a respiratory competent strain the glycerol-negative phenotype of our *pets* strain segregates 2:2 and is located 14 map units from the mating-type locus on chromosome 3. In crosses of the type *M*(o) *pets* x *M*(k) all respiratory-deficient progeny are nonkillers *M*(*o*), and sensitive. This result indicates that our killer, like Bevan's is unable to be maintained in the presence of *pets*, even though it is in the *M* background.

TABLE 2. *Meiotic segregation of the killer trait*

Genotype	Strains	Segregation K:NK	Number of tetrads
M(k) × *M*(o)	A8209B x A5107B	4:0	7
	A8205B x S288C	4:0	11
	A5164A x A8207B	4:0	9
M(k) × *m*(o)	A8205B x S18	2:2*	25
	A8209B x S18	2:2*	19
	A9545D x A8207B	2:2	10
M(k) × *M*(o) *pets*	A9193A x A8207B	2:2	10

These crosses are a small proportion of the large number performed to establish the segregation of the killer trait. The killer designation for each of the strains in Table 1 was determined by several crosses to *M*(*k*), *M*(o), and *m*(o) strains.

* In the *M*(k) × *m*(o) crosses with our killer strain, several *m*(k) spore clones were present. These were unstable and segregated *m*(o) at a high frequency. All autotrophic requirements in these crosses (*his, ade, trp, leu,* and *ura*) segregated 2:2, as expected for chromosomal genes. Only *ura3* had an effect on killer segregation.

Mitotic segregation of killer

Killer is extremely stable in the *M* strains during vegetative growth, although by screening a large number of haploid *M*(k) cells we have derived a few spontaneous nonkiller segregants *M*(o) from each of our standard killer strains. The killer strain was streaked twice, and a single clone shown to be *M*(k) was isolated, grown overnight in YEPD medium, diluted, and plated so that about 100 cells were spread on a YEPD plate. This plate was incubated at 30° for 2 days to allow the growth of cells into colonies, then replica plated to a sensitive indicator lawn. It was important to purify the killer strain before testing because prolonged storage on slants leads to a small, but significant, increase in haploid *M*(o) segregants. When purified *M*(k) clones are tested, *M*(o) segregants occur at a frequency of about 1 in 20,000 cells plated. We conclude that in the presence of the *M* allele the killer is extremely stable.

Curing of killer

M(k) strains were treated with inhibitors; among the inhibitors tested, only cycloheximide eliminates the killer trait effectively. To test the efficacy of a curing agent [a chemical that causes *M*(k) strains to be converted to *M*(o)], an overnight culture of cells grown from a pure clone of *M*(k) was diluted and plated on YEPD medium (as a control) and on YEPD plus inhibitor. The plates were incubated at 30° until colonies appeared, then replica plated to buffered YEPD plates on which lawns of a sensitive indicator strain had been spread. The appearance of killer clones can be scored in 24–48 hr. By trial, a concentration of inhibitor was determined that

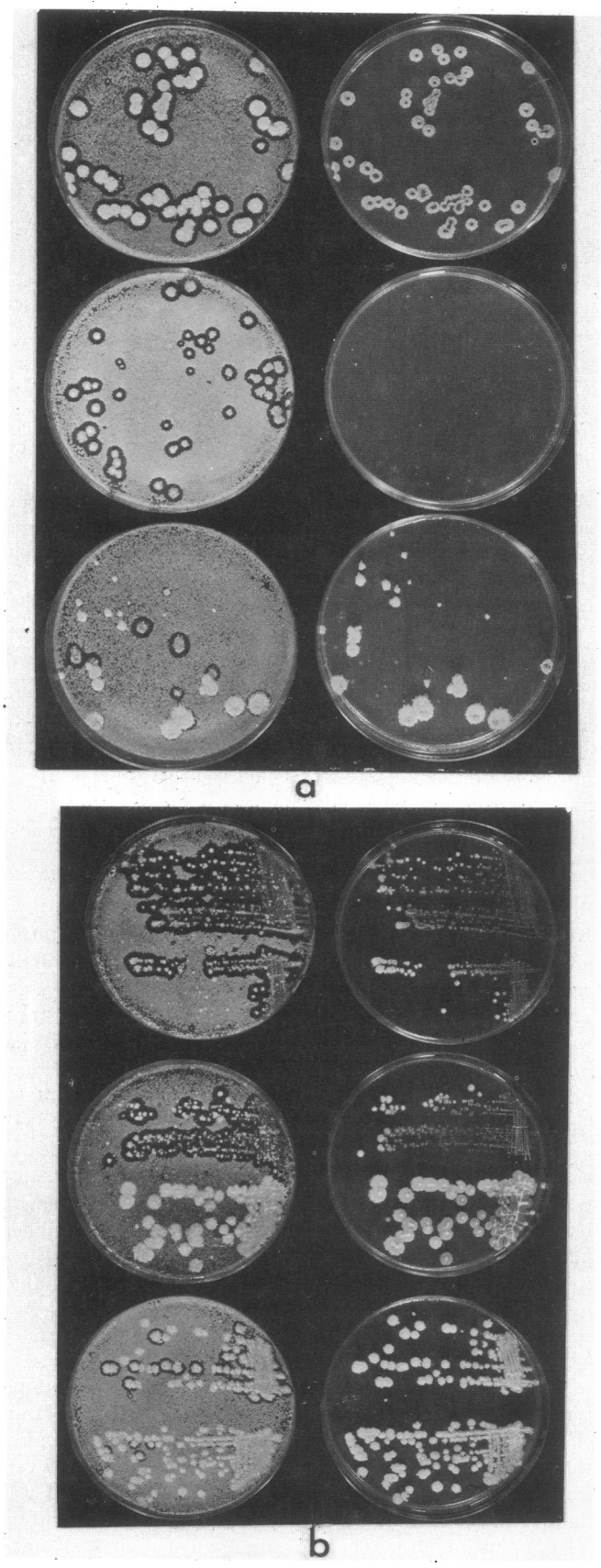

FIG. 1. (*a*) Cells of A8209B *M*(k) were plated on three different media: YEPD, YEPD + 1 mg per plate of ethidium bromide, or YEPD + 13.3 μg per plate of cycloheximide. Each of these was replicated to 1% yeast extract–2% peptone–3% glycerol medium to test for ρ^- strains (*right*) and to a lawn of a sensitive strain to test for killing (*left*). The replicates appear in the following order: No treatment (*top*), ethidium bromide (*center*), cycloheximide (*bottom*). The *dark area* encircling the colonies on the *left* plates is the zone of killing.

(*b*) Mixed colonies from cycloheximide-treated killer. Colonies that had been scored as *killer* in *a* were streaked on a YEPD plate (*right*) that was subsequently replicated to a sensitive lawn (*left*). "Killer" clones tested in this way usually segregated both *M*(*k*) and *M*(o) colonies. Thus, most of the clones that gave the appearance of having fully retained the killer trait after cycloheximide treatment were, in fact, mixed.

TABLE 3. *Curing of killer strains*

Drug	Amount added per plate (μg)	Strain	Colonies	% ρ^-	*M*(o)	Mixed *M*(k) + *M*(o)
Ethidium bromide	1000	A8209B ρ^+	804	100	0	0
	2000	A8209B ρ^+	376	100	0	0
Cyclohexamide	13.3	A8209B ρ^+	464	19	222	167
	13.3	A8209B ρ^-	74	—	1	73
	6.7	A8209B ρ^+	153	11	0	151
	3.3	A8209B ρ^+	228	4	0	143
Untreated	—	A8209B ρ^+	2686	3	0	0
	—	A8209B ρ^-	80	—	0	0

In the presence of cycloheximide, the conversion of *M*(k) to *M*(o) occurred at a high frequency. All killer strains tested were able to be cured with cycloheximide. Cycloheximide also increased the proportion of ρ^- clones, but loss of ρ did not coincide with loss of killer. Mixed clones were those that had obvious sectors or those that segregated *M*(k) and *M*(o) upon further testing.

was slightly less than that necessary to prevent growth. This "limit concentration" of inhibitor retarded, but did not completely inhibit, growth. In the curing experiment, this "limit concentration" and several lower dilutions were tested for their ability to cure the killer. The results are shown in Table 3 and Fig. 1*a*. Ethidium bromide converts all cells plated from ρ^+ to ρ^-, but causes no increase in frequency of *M*(k) to *M*(o).

We have performed two other experiments that indicate that ethidium bromide has negligible effects on the *M*(k) to *M*(o) conversion. First, to test whether treatment in liquid had a different effect, killer strains were grown in YEPD broth with 10 μg/ml of ethidium bromide for periods of 24–48 hr. This treatment has been reported to remove all detectable mitochondrial DNA (11). All clones tested were *M*(k) (ρ^-), that is, respiratory deficient and still killers. To test whether ethidium bromide had any effect on the stability of killer in spores, asci from a cross of *M*(k) x *m*(o) were dissected and germinated on agar containing ethidium bromide. All ascospore clones were ρ^-, showing the effectiveness of the ethidium bromide treatment. In each ascus the 2 *M* spore clones were killers and the 2 *m* spore clones were nonkillers.

Cycloheximide causes a dramatic increase in conversion of *M*(k) to *M*(o). In fact, nearly every clone tested after cycloheximide treatment is either completely *M*(o) or a mixed population of *M*(k) and *M*(o) (Fig. 1*b*). The cured *M*(o) is

still cycloheximide-sensitive, and has lost all three properties of the killer: ability to kill, immunity, and ability to contribute the killer in crosses. The killer determinant can be reintroduced in the cured strain as is shown by the 4:0 segregation $M(k):M(o)$ obtained when cured $M(o)$ is crossed by $M(k)$. $M(o)$ strains derived from cycloheximide treatment have not been observed to revert to killers. Acriflavine was tested in a similar system and caused a measurable, but less dramatic, increase in the frequency of conversion of $M(k)$ to $M(o)$.

DISCUSSION

Our studies show that many standard strains of yeast have the ability to kill other strains of yeast. This phenomenon has not been observed by most workers with yeast because the killing occurs only with media of low pH and not on standard petri plates. The killer strains we studied were strong killers and the sensitive strains were extremely sensitive nonkillers. Many other strains were either weak killers or less-sensitive nonkillers. In fact, the nonkiller $m(o)$ of Bevan used in this study was much less sensitive than our standard nonkiller strain. This range of sensitivity and killer potency probably reflects differences in both nuclear and cytoplasmic genetic backgrounds among these strains. Genetic crosses to identify these differences in background have not led to consistent segregation patterns that would allow resolution of these differences.

The curing studies with cycloheximide and ethidium bromide indicate that the killer is a cytoplasmic determinant capable of propagating in the absence of ρ. In fact, ρ^- killers appeared to kill better than ρ^+ killers in our petri plate test. Cycloheximide inhibits protein synthesis on cytoplasmic ribosomes (12), so it is unlikely that this drug has a direct effect on the killer genetic determinant. One possibility is that the cycloheximide treatment affects some protein (perhaps a replicase) synthesized on cytoplasmic ribosomes and necessary for the replication of the killer determinant. If it is assumed that this hypothetical protein is present in limiting amounts, then in the presence of cycloheximide the killer may be unable to replicate, even though the nuclear and mitochondrial genomes are able to do so. In subsequent cell division cycles the killer would be diluted out. It should be cautioned that our understanding of the conversion of $M(k)$ to $m(o)$ is limited by our detection system. It is possible that the killer genetic determinant is present in the $M(o)$ cells in a quiescent state such that none of the properties we attribute to the presence of the killer determinant are manifest.

The killer determinant could be any one of the nonnuclear nucleic acids that have been described in yeast and other fungi. Several laboratories have described a small circular DNA associated with a membrane fraction. This DNA has the buoyant density of nuclear DNA (1.701) and is present in ρ^- strains (13). No biological function has been ascribed to this DNA. Studies on the properties of an agent extracted from *Penicillium* that induces interferon have indicated that the active component (Statolon) is a double-stranded RNA of viral origin (14, 15). The viral particles can be collected from the filtrates or mycelia of *Penicillium* or *Aspergillus* cultures. A recent report claims that *Saccharomyces* can be infected with a double-stranded RNA virus isolated from *Aspergillus niger* (16, 17). There is, as yet, no diagnostic test indicative of the presence or absence of either of these nucleic acids. The identification of the killer nucleic acid should permit a deeper understanding of the molecular basis of the $M(k)$ to $M(o)$ conversion.

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