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Killer DNA Plasmids of the Yeast *Kluyveromyces lactis*

I. Mutations Affecting the Killer Phenotype

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Summary. The killer character of *Kluyveromyces lactis* is associated with a cytoplasmic genetic element which confers on the host cells: a) the capacity to kill sensitive cells of various yeast by producing a diffusible toxin, and b) the immunity to that toxin. The killing activity is associated with the presence of two linear DNA plasmids that we call k1 and k2. Both plasmids seem to be required for the expression of the killing activity. Mutants defective for the killing activity have been isolated by mutagenesis and are either cytoplasmic or nuclear. Four classes of cytoplasmic mutations have been found: 1) loss of k1; 2) loss of both k1 and k2; 3) internal deletion in k1 and 4) probable point mutations in the plasmids. Among the nuclear mutants, many conserved the normal plasmids, but expression of the killer phenotype was blocked. In some respects, the organisation of this plasmid system resembles a DNA version of the double-stranded RNA killer system of *Saccharomyces cerevisiae*.

Key words: Killer – *Kluyveromyces* – Plasmid

Introduction

The killer phenomenon is widespread among yeasts and yeast-like fungi. All the killer systems so far studied have been found to be associated with double-stranded RNA (dsRNA) plasmids.Gungé et al. (1981) first described the existence of two linear DNA plasmids associated with a killer phenotype in the yeast *Kluyveromyces lactis,* strain IFO 1267. We have found a killer activity in another strain, CBS 2359, which was correlated with the presence of two similar plasmids. The two plasmids found by Gungé et al. (1981) were named pGK1 1 and pGK1 2. As we will show, the plasmids from the two strains are indistinguishable in terms of killing spectra, plasmid structures and genetic properties. In this study, in which the two systems are examined in parallel, the plasmids will be called k1 (for the smaller plasmid, 8.8 kilobase-pairs long) and k2 (for the larger one, 13.4 kilobase-pairs long) and when, necessary, their origin will be specified by the name of the strain.

The two phenotypic traits, killing activity and immunity to it, show non-Mendelian inheritance. This DNA plasmid system recalls the well characterized killer system of *S. cerevisiae* which is associated with two dsRNAs. In that system, there exist chromosomal and non-chromosomal mutations which affect the killer character. Several classes of chromosomal mutations are known (see reviews: Bevan and Mitchell 1979; Bruenn 1980; Wickner 1981): *mak* mutations (*ma*intenance of *k*iller), *kex* mutations (*k*iller expression), *rex* (*re*sistance *ex*pression) and *ski* mutations (super *k*iller). Cytoplasmic mutations are also of several kinds: neutral (Somers and Bevan 1969), suppressive (Somers 1973), diploid-dependent (Wickner 1975) and plasmid mutations affecting maintenance (Toh-E and Wickner 1979).

In this paper, we report the isolation and characterization of *K. lactis* killer mutants. Some of them seem to be equivalent to the above mentioned *kex* mutants. However, no *mak* type mutations have been found, though they are numerous in the *S. cerevisiae* killer system. Different types of cytoplasmic mutations will be also described.

Materials and Methods

Notation. Some notation is inspired by those of the dsRNA killer system (Wickner and Leibowitz 1976). K^+ or K^- pheno-

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Table 1. List of *Kluyveromyces lactis* strains

Designation of strains	Killer phenotype	Genotype[a]	Plasmids		Source
			k1	k2	
IFO 1267	K^+R^+	*a* prototroph $[k1, k2]^{1267}$	+	+	N. Gungé
CBS 2359	K^+R^+	*a* prototroph $[k1, k2]^{2359}$	+	+	A. Algeri
1267/20	K^+R^+	*a trp*$^-$ $[k1, k2]^{1267}$	+	+	NG mutagenesis of IFO 1267
2359/152	K^+R^+	*a met*$^-$ $[k1, k2]^{2359}$	+	+	NG mutagenesis of CBS 2359
W600B	K^-R^-	α *ade1 ade2 leu*$^-$ $[k1^\circ, k2^\circ]$	–	–	A. D. Panek
KA5-6C	K^-R^{+}*	α *ade2 his*$^-$ *leu*$^-$ $[k1^\circ, k2^\circ]$	–	–	A. D. Panek* see text for this resistance character
1267/20/VH1 (or VH1)	K^-R^-	*a trp*$^-$ $[k1^\circ, k2^\circ]$	–	–	UV mutagenesis of 1267/20
2360/7	K^-R^-	α *lys*$^-$ $[k1^\circ, k2^\circ]$	–	–	A. Algeri
1267/NK2 (or NK2)	K^-R^+	*a* prototroph [*k1-NK2, k2*]	modified k1	+	Spontaneous mutant
1267/NK2/1 (or NK2/1)	K^-R^+	*a his*$^-$ [*k1-NK2, k2*]	id	+	NG mutagenesis of 1267/NK2 to introduce a *his*$^-$ marker
MW6-4C	K^-R^+	α *ade2 his*$^-$ [k1-NK2, k2]	id	+	meiosis of 1267/NK2/1 x MW1.8C
MW1-8C	K^+R^+	α *ade2* $[k1, k2]^{1267}$	+	+	meiosis of W600B x 1267/20
MW26-3D	K^+R^+	α *lys*$^-$ $[k1, k2]^{2359}$	+	+	meiosis of 2359/152 x 2360/7
MW3-3A	K^-R^-	*a lys*$^-$ $[k1^\circ, k2^\circ]$	–	–	meiosis of KA5-6C x 2360/7

[a] The small superscripts 1267 and 2359 indicate that the cytoplasm comes from IFO 1267 and CBS 2359 respectively

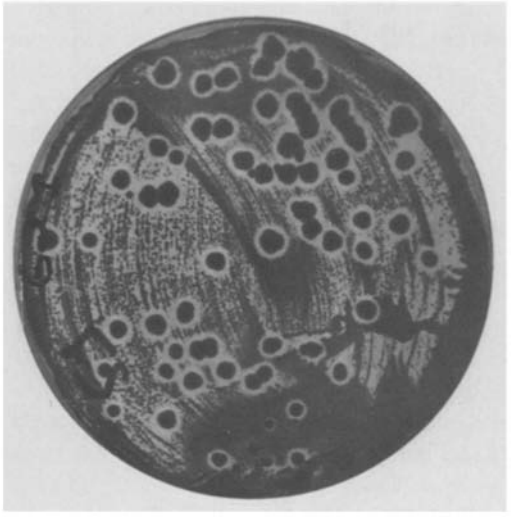

Fig. 1. Killer test. Colonies growing on complete glucose medium are transferred by replica-plating to a lawn of a sensitive strain to test for killing, as described in Materials and Methods. The plates were incubated at 28 °C

type stands for ability or inability of a strain to kill sensitive tester strains. R^+ or R^- phenotype refers to resistance or sensitivity to killer toxin. The chromosomal mutations which are required for killer expression by the wild-type plasmids are designated by *kex*. The cytoplasmic genotypes are indicated in square brackets as follows. [*k1, k2*]: presence of wild-type k1 and k2 plasmids; [*k1*°, *k2*]: k1 is absent, k2 is wild-type; [*k1-x, k2*]: mutation x (H105, NK2, etc.) in k1, k2 being wild-type; [*k1*°, *k2*°]: absence of k1 and k2. Better notation may be defined when we know more about this new system.

Strains. Yeast strains used are listed in Table 1.

Media and Culture Conditions. The complete glucose medium contained 2% glucose, 1% Difco yeast extract and 1% Difco bactopeptone. The Gall medium contained 2% galactose, 1% yeast extract, 1% bactopeptone and 0.05 M potassium dihydrogen phosphate pH 4.5 as such. The minimal medium contained 2% glucose and 0.7% Difco yeast nitrogen base (without aminoacids). For plating, these media were supplemented with 2.3% Difco Bactoagar. Mating and sporulation medium ME contained 5% Difco Bacto-malt extract and 3% Bactoagar. The temperature for all cultures was 28 °C.

Assay of Killing and Resitance. Colonies to be tested for killing activity were replicated onto a lawn of the sensitive tester strain 2360/7 on Gall plates. Resistance to killing was assayed by replicating the colonies of the tester killer strains IFO 1267 or CBS 2359 onto a lawn of the strain to be tested on the Gall plates. After 2 days incubation, killing was indicated by a clear zone surrounding the killing colonies (Fig. 1). The complete glucose medium or less acidic media were found to be unfavorable for this test under our conditions.

Mutagenesis. Ethyl-methanesulfonate (EMS) and N-methyl-N^1-nitrosoguanidine (NG) were used for the isolation of auxotrophic

mutants. EMS, NG, ultraviolet as well as $MnCl_2$ were used to induce non-killer mutants. *(a) EMS mutagenesis.* Cells grown to about 10^8 cells/ml in complete glucose medium were washed and diluted in Ringer solution at 10^7 cells/ml. 0.2 ml of the suspension was mixed with 1.5 ml of 0.1 M sodium phosphate buffer, pH 6.8, and 50 µl of EMS. After incubation at 28 °C for 60 min, cells were diluted in 5% sodium thiosulfate for plating; *(b) NG mutagenesis.* To exponential phase cells in complete glucose medium, a solution of NG (3 mg/ml acetone) was added to a final concentration of 12–60 µg/ml. After incubation for 45–120 min, cells were diluted in Ringer solution and plated; *(c) $MnCl_2$ mutagenesis.* 10^8 cells were inoculated into 2 ml of complete glucose medium and grown for 18 h in the presence of 10^{-2} M $MnCl_2$, then plated after appropriate dilution; *(d) ultraviolet irradiation.* Stationary phase cells were suspended in Ringer at 10^8 cells/ml and irradiated under a germicidal lamp at a flux of 1 Joule · m^{-2}. s^{-1} for various lengths of time.

The primary clones of mutants were all subcloned and single clones were retained. Non-killar mutants were subsequently tested for resistance to killing.

Genetic Analysis. Conjugation, sporulation and tetrad analysis were performed according to Tingle et al. (1968) and Brunner et al. (1977). Haploid strains of opposite mating type and complementary auxotrophy were mated en masse on the ME plates at 28 °C. After 48 h, zygote clones were diluted and plated on minimal medium. After 2–3 days at 28 °C, the prototrophic diploids were replicated onto ME plates, allowed to grow for 24 h at 28 °C, then stored overnight at 4 °C for sporulation. Colonies were examined under a microscope for the presence of asci. Dissection of asci was performed after digestion of the cell wall with glusulase.

Detection of Plasmid DNA. Plasmid DNA was prepared from early stationary phase cells grown in 200 ml of the medium containing 2% galactose, 0.1% glucose, 1% yeast extract and 1% bacto peptone. Total DNA was extracted essentially according to Gungé et al. (1981). The DNA was fractionated by electrophoresis on 0.6% agarose (Biorad), using 40 mM Tris-acetate buffer, pH 8, containing 1 mM EDTA. DNA bands were detected by ethidium bromide staining (0.5 µg/ml, 3 h) and photographed under ultraviolet light through a red filter.

Results

Original Killer Strains and their Derivatives

Starting from the two original killer strains, IFO 1267 and CBS 2359, two separate genetic systems have been constructed. Although the plasmids from the two strains appeared to be identical in all aspects examined, we avoided, for formal reasons, to mix them by crosses. Auxotrophic markers were introduced into the original prototrophic strains by direct mutagenesis rather than crossing with current laboratory strains except for a few cases, since some of them contained nuclear genes conferring resistance to the killer. The strain KA5-6C from Dr. A. D. Panek, for instance, contained a single nuclear gene for resistance which could be eliminated through meiosis. Our killer-sensitive reference strains are 2360/7, MW3-3A and VH1 which have no detectable plasmids and are fully sensitive to the original killer strains. When they were crossed with killer $0K^+R^+$ strains, all the tetrads obtained were $4(K^+R^+):(K^-R^-)$. The plasmids of the original *a* mating type strains were transferred to α mating type cells through crosses with plasmid-less strains.

Isolation on Non-killer Mutants

Four auxotrophic killer strains were used for the isolation of non-killer mutants: 1267/20 (*a trp*–) and

Table 2. Mutagenesis

Mutagen	Dose	Strain	% Survival	Colonies tested	Total number of auxotrophic mutants	Total number of non-killer clones	Mutants used in this study	Name of non-killer series
EMS	34 µg/ml 60 min	IFO 1267	60	1,000	3	0	1267/20 (trp^-)	–
NG	12 µg/ml, 2 h	CBS 2359	75	1,200	10	not scored	2359/152 (met^-)	–
	30 µg/ml, 60 min	NK2	50	3,000	63	63	NK2/1 (his^-)	–
	12 µg/ml, 45 min	1267/20	75	4,000	not scored	38	all	H
	60 µg/ml, 80 min	MW1-8C	10	20,000	not scored	31	all	M
Ultraviolet	60 Joule · m^{-2}	1267/20	25	6,000	not scored	3,000	VH1	VH
	20 Joule · m^{-2}	2359/152	8.6	10,000	not scored	8	all	VA
	10 and 20 Joule · m^{-2}	MW26-3D	60 and 20	5,000	not scored	9	all	VM
	60 → 100 Joule · m^{-2}	MW26-3D	variable	1,000	not scored	26	4 K^-R^+clones (VP21,VP22,VP25, VP35)	VP
Mn^{2+}	$MnSO_4$ 10^{-2} M, 18 h	MW26-3D	slow growth	2,500	not scored	17	K16	K

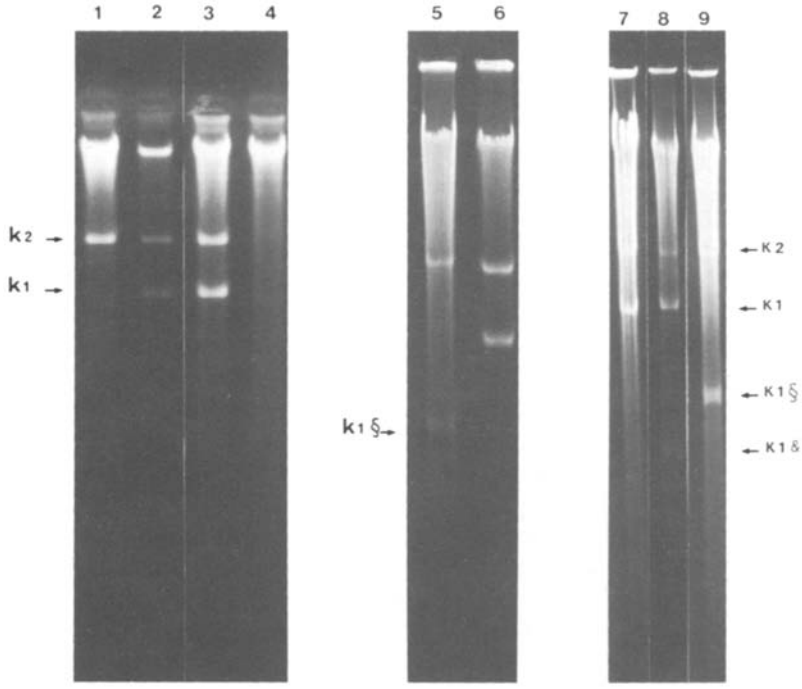

Fig. 2. Plasmid composition of killer and its mutants. Extracts were subjected to electrophoresis on 0.6% agarose gels. The gels are stained with ethidium bromide.
1. Loss of k1; 2. Wild-type k1 and k2; 3. non killer plasmids of apparently normal sizes; 4. Loss of both k1 and k2; 5. k1 is replaced by a smaller plasmid (k1§); 6. and 7. Wild-type k1 and k2; 8. Non killer mutant with a third, small plasmid in addition to apparently normal k1 and k2: the third plasmid may have two components. 9. identical to 5

MW1-8C (α *ade*−) for the IFO 1267 plasmids series, and 2359/152 (*a met*−) and MW26-3D (α *lys*−) for CBS 2359 plasmids series. The results of mutagenesis are summarized in Table 2. A rather limited number of non-killer mutants were obtained by chemical mutagens. Ultraviolet irradiations at high doses sometimes gave an unusually high number of mutants (up to 50%) most of which, however, have lost both plasmids.

All the mutants obtained were either K^-R^- or K^-R^+. A total of 92 mutants have been analyzed by genetic crosses and by electrophoresis of their plasmids.

Non-killer mutants fell into five categories with respect to their plasmid composition (see Fig. 2): 1) loss of k1; 2) loss of both k1 and k2; 3) plasmids of apparently normal sizes; 4) k1 is replaced by a smaller plasmid and 5) presence of a third, small plasmid in addition to apparently normal k1 and k2 (Table 3).

Mutants That Have Lost k1 or Both k1 and k2

These mutants were quite frequent and found in all types of mutagenesis and 53 were obtained. They generally had a K^-R^- phenotype (Table 3). When they were crossed to a wild-type killer strain, more than 95% of the diploids were K^+R^+ and the tetrads from crosses involving 51 of the K mutants segregated $4(K^+R^+):0(K^-R^-)$ indicating the absence of a nuclear mutation. The remaining two mutants, M16 and H109 contained no k1 plasmid, retaining apparently normal k2. When crossed to K^+R^+ strains, all diploids were K^+R^+, but they segregated into $2(K^+R^+):2(K^-R^-)$ tetrads; all four spores possessed k1 and k2. This means that the two mutants have double mutations: loss of k1 on one hand, and a recessive nuclear mutation which affects the expression of killer activity of normal plasmids, on the other. We call this nuclear mutation *kex* by analogy to the dsRNA killer system (see below).

Mutants with No Apparent Alteration of k1 and k2 Plasmids

We have found many non-killer mutants which have nevertheless conserved apparently normal plasmids. They were all K^-R^+. Thirty-six out of 38 of these mutants could restore the K^+R^+ phenotype when crossed to plasmid-less K^-R^- testers; the diploids from such crosses gave a spore segregation of $2(K^+R^+):2(K^-R^+)$ (Table 3). This indicated the presence of a recessive chromosomal mutation of the *kex* type. These thirty-six *kex* mutants, together with the above mentioned two *kex* mutants (M16 and H109), all fell into a single complementation group that we named *kex1*.

In contrast, the remaining two K^-R^+ mutants (H82 and VA3) did not restore the K^+R^+ phenotype when crossed to the plasmid-less testers. The tetrads from such crosses segregated $4(K^-R^+):0(K^-R^-)$. When the mutants were crossed to K^+R^+ strains, both K^+R^+ and K^-R^+ diploids were obtained with about the same frequency. Meiosis of the K^+R^+ diploids yielded all possible ratios of K^+R^+ and K^-R^+ spores, from 4:0

Table 3. Meiotic analysis of non-killer mutants

Mutant	Killer phenotype	Plasmids[b,c] k1/k2	Diploid phenotype when crossed to		Plasmids of diploid k1/k2	Meiotic segregation		Remarks
			K^+R^+	K^-R^-		Killer phenotype	Plasmids	
H105, M33, VM8...	K^-R^-	−/−	K^+R^{+a}		n.d.	$4K^+R^+:0$	4(+/+):0	cured of both k1 and k2
H85, M47, VM29	K^-R^-	−/+	K^+R^{+a}		n.d.	$4K^+R^+:0$	4(+/+):0	cured of k1
M16 and H109	K^-R^-	−/+	K^+R^{+a}		n.d.	$2K^+R^+:2K^-R^-$	4(+/+):0	*kex* and cured of k1
H87, M7, VA8	K^-R^+	+/+		K^+R^{+a}	n.d.	$2K^+R^+:2K^-R^-$	4(+/+):0	*kex*
H82 and VA3	K^-R^+	+/+		K^-R^+	+/+	$4K^-R^+:0$	4(+/+):0	
			K^+R^+		n.d.	$K^+R^+:K^-R^+$ (all possible ratios see 3rd paper for details)	4(+/+):0	Probably point mutations of plasmids
			and					
			K^-R^+		+/+	$4K^-R^+:0$	4(+/+):0	
NK2/1	K^-R^+	§/+	K^+R^+		§/+	$4K^+R^+:0K^-R^+$ (1) $3K^+R^+:1K^-R^+$ (1) $2K^+R^+:2K^-R^+$ (2) $1K^+R^+:3K^-R^+$ (2) $0K^+R^+:4K^-R^+$ (20)	+/+:§/+[e]	internal deletion of k1[d]
			and					
			K^-R^+		n.d.	$4K^-R^+:0$	4(§/+):0	
				K^-R^+	§/+	$4K^-R^+:0$	4(§/+):0	
VM5	K^-R^-	&/+/+	K^+R^+		+/+	$4K^+R^+:0$	4(+/+):0	origin of the third
				K^-R^-	−/−	$4K^-R^-:0$	4(−/−):0	plasmid is unknown

[a] In these crosses about 1% of diploid clones were of K^-R^- phenotype; when meiotic analysis of such a diploid was performed the segregation was of $4K^-R^-:0$ type which indicates that cytoplasmic mixing had not occurred

[b] Presence (+) and absence (−)

[c] §, &: size modified

[d] Four other mutants, H7, H22, H23 and H24 showed a similar deletion of k1

[e] K^+R^+ spores contained both wild-type plasmids; K^-R^+ spores contained k2 and size modified plasmid. The numbers in the parentheses represent the number of tetrads analyzed

to 0:4. Meiosis of K^-R^+ diploids gave $4(K^-R^+):0(K^+R^+)$ segregation. In all cases, spores contained k1 and k2 of normal size.

The simplest interpretation of these data is that H82 and VA3 carry a cytoplasmic point mutation. These two mutants will be examined more in detail by Wésolowski et al. (1982b).

Mutants with Large Alterations of Plasmids

a) Deletion Mutants. The non-killer K^-R^+ mutant NK2 is a spontaneous mutant isolated from the original IFO 1267 strain. The mutant has an apparently normal k2 plasmid but k1 is replaced by a smaller plasmid (5.9 kbp). Four similar mutants (H7, H22, H23, H24) have also been isolated from the same stock. NK2/1 is an auxotrophic subclone isolated from NK2, which conserved the same plasmid pattern. The results of crosses between NK2/1 and a K^-R^- strain (Table 3) indicated that the mutation is inherited cytoplasmically. Restriction enzyme analysis of the 5.9 kbp plasmid and hybridization experiments have shown that this DNA derived from k1 by an internal deletion of 2.9 kbp (Wésolowski et al. 1982a). This suggests that the deleted part of the k1 DNA is required for the toxin production.

The occurrence of such a deletion in k1 DNA can be compared to the deletions found in the 'suppressive' M dsRNA of the *S. cerevisiae* killer system (Fried and Fink 1978). When NK2/1 was crossed to a wild-type K^+R^+ strain, the diploid population issued from the cross showed a strong bias in favour of K^-R^+ cells (75–80%). This is quite different from the standard cross K^+R^+ x no plasmid K^-R^- in which more than 99% of diploids were K^+R^+. It is not known whether the short NK2/1 genome has any selective advantage over the normal k1 DNA. Also, the input ratio of the parental cytoplasms in the zygotes may vary considerably

for different mutants. The mitotic segregation of the K^+R^+ diploids from the above cross is complex. On subcloning, they gave rise to K^-R^+ cells with a frequency as high as 35% to over 50%. K^-R^+ diploids fom the same cross remained pure K^-R^+. One interpretation is that the mutant k1 and the wild-type k1 genomes coexist in the diploid cells and that these heteroplasmic cells purify to an homoplasmic state after a large number of cell divisions (20–30 generations). Since most of the heteroplasmic cells carrying both killer and non-killer plasmids are likely to be scored as killers, the early K^+R^+ clones will segregate out non-killers during vegetative growth.

The high frequency of K^-R^+ cells in the cross NK2/1 x K^+R^+ may suggest the existence of phenomenon similar to the suppressiveness of rho$^-$ mutants of *S. cerevisiae*. We are investigating whether such a property can be defined in this system. In dsRNA killer system, cytoplasmic deletion mutants are named suppresive.

b) Three Plasmids Mutants. The mutant VM5, obtained after ultraviolet irradiation, has lost the ability to kill and the resistance to toxin, but maintained apparently normal k1 and k2 DNA. It contained, in addition, a third small plasmid of about 5 kbp. The three plasmids are carried by the same cells since they are stably transmitted together through subclonings. On crossing VM5 with a plasmid-less K^-R^-, all the diploid clones obtained were K^-R^- and no plasmids were detected in them. When VM5 was crossed to K^+R^+, all the diploids obtained were K^+R^+. Also, crossing VM5 with the deletion mutation NK2/1 or with a mutant carrying only the k2 plasmid did not restore the killer activity. Everything appears as if cytoplasmic mixing could not occur and the cytoplasm of VM5 was excluded in the crosses. The origin of the 5 kbp plasmid is under investigation.

Discussion

Killer activity of *K. lactis* seems to require the simultaneous presence of k1 and k2 plasmids, although only k1 has been related unambiguously to the killer phenotype. In fact, all the killer strains harbor the two plasmids. While k2 can be maintained alone in non-killer strains, k1 has never been observed in the absence of k2. These facts suggest a dependence of k1 on the presene of k2. As yet, no non-killer mutations have been directly related to k2. An equivalent situation is known in the dsRNA killer system of *S. cerevisiae*, that is , a dependence of the small M-dsRNA on the large L-dsRNA. The L-dsRNA codes for a capsid polypeptide (Hopper et al. 1977) necessary to form both L and M virus-like particles, while M-dsRNA codes for the toxin. It is tempting to postulate that k1 and k2 DNA occur in the form of such virus-like particles, but no experimental data are available on this point.

Our results show that the killing activity and the immunity are borne by k1 DNA and inherited in a non-Mendelian manner. Amongst ninety two non-killer mutants examined, only one type of nuclear mutation affecting the killer character has been demonstrated (*kex1*). Despite similarities in many respects with the dsRNA killer system, no nuclear mutations affecting the maintenance of killer (equivalent of *mak*) have been found. The killer DNA of *K. lactis*, which is much larger than the killer dsRNA, may code for essential functions for its own maintenance. However, the low buoyant density in CsCl of the plasmid DNA (Gungé et al. 1981) suggests that this genome contains a high proportion of adenine-thymine sequences, which limits the coding capacity of the genome as in the case of yeast mitochondrial DNA. Our failure to find *mak* type mutations in *K. lactis* may also suggest that the killer activity of *K. lactis* can be of "super-killer" form described for *S. cerevisiae* which can bypass many *mak* mutations (Toh-E and Wickner 1980). In the killer system of *Ustilago maydis*, no nuclear genes concerning maintenance or expression have been described (Koltin 1977; Koltin and Day 1976).

We have shown in this paper that it is possible to obtain various types of mutations affecting the killer activity. Also as shown by Wésolowski et al. (1982b), genetic recombination can be observed between plasmid mutants. Thus, the *K. Lactis* killer system appears to be suitable for detailed genetic and biochemical analyses. Gungé and Sakaguchi (1981) have been successful in transferring k1 and k2 plasmids into *S. cerevisiae*. A direct comparison between the dsRNA killer system and the DNA killer system in the same host cells is therefore possible.

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