A MUTANT KILLER PLASMID WHOSE REPLICATION DEPENDS ON A CHROMOSOMAL "SUPERKILLER" MUTATION

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ABSTRACT

Yeast strains carrying a 1.5×10^6 molecular weight linear double-stranded RNA in virus-like particles (*M* dsRNA, the killer plasmid or virus) secrete a toxin that is lethal to strains not carrying this plasmid. Recessive mutations in any of four chromosomal genes (called *ski1-ski4*) result in increased production of toxin activity. We report here a mutation of the killer plasmid (called [KIL-sd] for *ski-d*ependent) that makes the killer plasmid dependent for its replication on the presence of a chromosomal mutation in any *ski* gene. Thus, the [KIL-sd] plasmid is lost from *SKI+* strains. When the wild-type killer plasmid, [KIL-k], is introduced into a *ski2–2* [KIL-o] strain, the killer plasmid changes to a [KIL-sd] plasmid. This may represent a specific form of mutagenesis or selective replication in the *ski2–2* strain of [KIL-sd] variants (mutants) in the normal [KIL-k] population. The *ski2–1* and *ski2–3* mutations do not convert [KIL-k] to [KIL-sd], but *ski2–3* does allow maintenance of the [KIL-sd] plasmid. The [KIL-sd] plasmid thus lacks a plasmid site or product needed for replication in wild-type cells.

YEAST strains carrying a 1.5×10^6 dalton double-stranded RNA plasmid (*M* dsRNA) secrete a protein toxin (the killer toxin) that is lethal only to strains not carrying this plasmid (MAKOWER and BEVAN 1963; SOMERS and BEVAN 1969; BEVAN and SOMERS 1969; WOODS and BEVAN 1968; BUSSEY, 1972; BEVAN, HERRING and MITCHELL 1973; VODKIN, KATTERMAN and FINK 1974). The *M* dsRNA is found in intracellular virus-like particles (BUCK, LHOAS and STREET 1973; HERRING and BEVAN 1974; ADLER, WOOD and BOZARTH 1976).

At least 27 chromosomal genes are needed for killer-plasmid maintenance. These are *mak1* through *mak8* (WICKNER 1974a; WICKNER and LEIBOWITZ 1976a), *mak10* (SOMERS and BEVAN 1969), *mak9* and *mak14* (LEIBOWITZ and WICKNER, unpublished), *mak11*, *mak12*, *mak13*, and *mak15* through *mak25* (WICKNER 1978), *pet18* (FINK and STYLES 1972; HAWTHORNE and MORTIMER, unpublished; LEIBOWITZ and WICKNER 1978), and *spe2* (COHN *et al.* 1978).

Four chromosomal genes, *ski1* through *ski4*, negatively control toxin production by killer strains; recessive mutations in these genes result in overproduction of killer toxin activity, possibly by an effect on killer plasmid replication (TOH-E, GUERRY and WICKNER 1978).

Previously described killer plasmid mutations include neutral [KIL-n] mutants (SOMERS and BEVAN 1969), defective interfering ([KIL-s] suppressive) mutants (SOMERS 1973; VODKIN, KATTERMAN and FINK 1974; SWEENEY, TATE and FINK 1976), diploid-dependent [KIL-d] mutants (WICKNER 1976), superkiller [KIL-*ski*] plasmid mutants (VODKIN, KATTERMAN and FINK 1974; SWEENEY, TATE and FINK 1976), and immunity defective [KIL-i] mutants (VODKIN, KATTERMAN and FINK 1974; SWEENEY, TATE and FINK 1976).

In this communication we describe mutants of the killer plasmid, called [KIL-sd], whose replication is dependent upon the presence of a chromosomal *ski* mutation. These plasmid mutants lack a killer genome-coded function essential for its own maintenance and replication.

MATERIALS AND METHODS

Media: YPAD, SD, YPG, MB, presporulation, sporulation, complete minimal, and omission media were described previously (WICKNER and LEIBOWITZ 1976b). Neutralized YPAD was prepared by adding 3 ml of 1 M potassium phosphate, pH 7.5, to 100 ml of YPAD (final pH about 7.0).

Assay of killing and resistance: These assays were performed at either 20° or 30° as previously described (WICKNER and LEIBOWITZ 1976b).

Definition of superkiller: Superkillers are those strains producing a clear zone of killing at 30°, a temperature at which wild-type killers produce almost no killing (TOH-E, GUERRY and WICKNER 1978).

Strains: Yeast strains used in this study are listed in Table 1. Phenotypes of strains with regard to their killing (K) and resistance (R) to killing are denoted K+R+, K-R+, K+R-, and K-R-. These can arise as a result of many different genotypes. K++ denotes the superkiller

TABLE 1

Yeast strains

Strain designation	Killer phenotype	Genotype
AN33	*K-R-*	α *arg1 thr1* [KIL-o]
53	K-R-	**a** *ade1* [KIL-o]
AT129	K+R+	**a** *leu1 kar1-1* [KIL-k]
W9-14D	K++R+	**a** *ade1 his7 ski2-2* [KIL-sd1]
1028	K-R-	**a** *can*^{r} *cyh2 ade5 met13 trp5* ρ° [KIL-o]
1029	K-R-	α *can*^{r} *cyh2 tyr1 lys2 mak5-1* ρ° [KIL-o]
AT185	K-R-	**a** *leu1 kar1-1 ski2-2* [KIL-o]Δ ρ°
AT197	K++R+	**a** *leu1 lys10 kar1-1 ski2-2* [KIL-sd1]
W89-1A	K++R+	α *arg1 thr1 ski2-2* [KIL-sd1]
W89-1C	K++R+	α *ade1 ski2-2* [KIL-sd1]
AT158	K-R-	α *ade1 ski2-2* [KIL-o]Δ
AT211	K-R-	α *thr1 ski2-3* [KIL-o]Δ
AT213	K-R-	α *arg1 ski3-3* [KIL-o]Δ
AT215	K-R-	α *arg1 thr1 ski4-1* [KIL-o]Δ
AT220	K-R-	α *his7 ski1-1* [KIL-o]Δ
5X47	K-R-	**a**/α *his1*/+ *trp1*/+ *ura3*/+ [KIL-o]
200	K-R-	**a** *lys10* [KIL-o]
201	K-R-	α *lys10* [KIL-o]

phenotype. In some crosses, K+ and K++ were not distinguished. Curing of the killer plasmid is done by growing killer strains at 37° (WICKNER 1974b). Killer plasmid genotype of *ski*-dependent mutants is indicated as [KIL-sd1], [KIL-sd2], or [KIL-sd3]. The number is an allele number. Plasmid genotypes of cured strains are written as [KIL-o]Δ. Curing is facilitated by first growing the strain to be cured in neutralized YPAD broth at 37° and then plating appropriate dilutions on YPAD plates that are incubated at 37°. Mitochondrial DNA was eliminated from strains by streaking to single colonies on YPAD containing 30 μg per ml ethidium bromide.

RNA extraction and agarose gel electrophoresis: RNA was extracted either from spheroplasts as previously described (WICKNER and LEIBOWITZ 1976b; TOH-E, GUERRY and WICKNER 1978) or from intact cells as described by FRIED and FINK (1978). Electrophoresis was done on agarose gels (1%), and photographs were taken as described previously (TOH-E, GUERRY and WICKNER 1978).

Cytoduction: A cytoplasmic genome can be transferred from one strain to another without diploidization by heterokaryon formation using the *kar1* mutant, defective in nuclear fusion, described by CONDE and FINK (1976). Usually recipient cells were ρ°, and donor cells were ρ+. After mating recipient and donor in neutralized YPAD overnight, donor nuclei were counterselected by plating the mating mixture onto appropriate media. Respiratory competent cells having recipient nuclei were cytoductants.

RESULTS

Identification of the [KIL-sd] mutation: Ski mutants overproduce the killer toxin due to recessive mutations in one of four chromosomal genes (TOH-E, GUERRY and WICKNER 1978). When one *ski2–2* strain (W9–14D) was crossed with AN33 (+ [KIL-o]), most tetrads unexpectedly showed 2 K++ : 2 K- segregation, with occasional 1 K++ : 3 K- and 0 : 4 K- tetrads (Table 2, cross W89). This segregation pattern was completely different from the 2 K++ : 2 K+ pattern previously observed for all crosses of the type *ski*- [KIL-k] × + [KIL-o] (TOH-E, GUERRY and WICKNER 1978), K- segregants from cross W89 were sensitive to killer toxin. To determine whether the K-R- segregants in cross W89 had lost the killer plasmid, several such segregants were crossed with a standard + [KIL-o] strain and the resulting diploids were scored for the killer trait. Since these diploids were mostly non-killers in each case, the K-R- phenotype of the segregants described above was due to the loss of the killer plasmid (see also below).

These K-R- segregants were capable of maintaining the normal killer plasmid because crossing either of two of them (W89–1D and W89–2B) with the wild-type killer strain S37 resulted in only 4 K+ : 0 segregation (Table 2). Since all of the K+ segregants in cross W89 were K++, the *ski2*+ nuclear background must be unable to maintain the particular killer plasmid in this cross. Thus, the killer plasmid present in the *ski2–2* parent (W9–14D) was lost in the *SKI*+ segregants of this cross, but the same segregants could maintain the wild-type killer plasmid. This result suggests that a mutation of the killer plasmid in the *ski2–2* strain occurred such that the mutant killer plasmid has become dependent on the *ski2–2* mutation for its maintenance. This putative mutant killer plasmid is denoted [KIL-sd1] for *ski*-dependent.

Two further experiments are consistent with this interpretation: (1) crosses between either of two K++ segregants, W89–1A and W89–1C, and K- segregants of W89 gave essentially the same segregation pattern as that of cross W89 and

TABLE 2

Tetrad analysis of ski2–2/+ *diploids*

			Segregation				
			4 K+:0	3 K+:1 K⁻	2 K+:2 K⁻	1 K+:3 K⁻	0:4 K⁻
W89	AN33	α *thr1 arg1* + + [KIL-o]	0	0	18	1	0
	W9–14D	**a** + + *ade1 ski2–2* [KIL-sd1]*					
W128	W89–1A	α *arg1 thr1 ski2–2* + + [KIL-sd1]	0	1	12	1	1
	W89–1B	**a** *arg1* + + *ade1 his7* [KIL-o]					
W129 W132	W89–1C	α *ade1* + *ski2–2* [KIL-sd1]	1	0	26	3	2
	W89–2B	**a** + *thr1* + [KIL-o]					
W117	S37	α *leu2 met5* + + [KIL-k]	15	1	0	0	0
	W89–1D	**a** + + *his7 thr1* [KIL-o]					
W118	S37	α *leu2 met5* + [KIL-k]	19	1	0	0	0
	W89–2B	**a** + + *thr1* [KIL-o]					
W164	AT129	**a** *leu1 kar1* + + [KIL-k]	17 (2 *ski* : 2 wild-type)	1	0	0	0
	W95–13A	α + + *lys10 ski2–2* [KIL-sd1]					
W327	AT193–1	α *ade1* + + *ski2–2* [KIL-sd2]*	0	0	16	2	0
	W89–1D	**a** + *his7 thr1* + [KIL-o]					
W295	AT193–2	α *ade1* + + *ski2–2* [KIL-sd3]*	0	0	18	2	0
	W89–1D	**a** + *his7 thr1* + [KIL-o]					

* [KIL-sd1] indicates the first mutant [KIL-sd] plasmid isolated. [KIL-sd2] and [KIL-sd3] are independent isolates of the same type of mutant (see text). In this table, K+ means either K++ or K+.

(2) the cross *ski2–2* [KIL-sd1] × *SKI*+ [KIL-k] (cross W164 in Table 2) showed essentially 2 K++ : 2 K+ segregation.

Since the *ski2–2* mutation is recessive, the explanation mentioned above can be tested by investigating the killer phenotype of *ski2 / SKI*+ [KIL-sd1] heterozygous diploids. Diploids were obtained by crossing a *ski2–2* [KIL-sd1] strain with + [KIL-o] or *ski2–2* [KIL-sd1] or *ski2–2* [KIL-o] strains, and the diploids were streaked onto SD plates to generate single colonies. The killer phenotype of each colony was scored (Table 3 and Figure 1). Homozygous diploids, *ski2–2 / ski2–2* [KIL-sd1], can maintain the plasmid stably, whereas heterozygous diploids, *ski2–2 / SKI*+ [KIL-sd1], easily loose the plasmid. However, the fraction of K+ colonies in the heterozygous diploids is variable, depending on how long the diploids have grown. Thus, diploids sporulated soon after formation (as in Table 2) show only a few 4 K⁻ : 0 tetrads, while those grown to single colonies after formation show mostly 4 K⁻ : 0 segregation. This mitotic loss of

TABLE 3

Elimination of [KIL-sd] plasmid from ski2–2/+ *[KIL-sd] diploid*

Diploid		Single colonies tested	No. of K+
W89–1C	*α ade1 + + ski2–2* [KIL-sd1]	60	60
W89–5B	**a** *arg1 thr1 his7 ski2–2* [KIL-sd1]		
W89–1C	*α ade1 + + + ski2–2* [KIL-sd1]	30	30
W164–11BΔK⁻	**a** + *leu1 lys10 kar1 ski2–2* [KIL-o]		
W89–1C	*α ade1 + ski2–2* [KIL-sd1]	13	1 (weak)
W89–2B	**a** + *thr1* + [KIL-o]		
W89–1A	*α arg1 thr1 + + ski2–2* [KIL-sd1]	55	1 (weak)
W89–1B	**a** *arg1* + *his7 ade1* + [KIL-o]		
W89–1A	*α arg1 thr1 + ski2–2* [KIL-sd1]	55	1 (weak)
W89–1D	**a** + *thr1 his7* + [KIL-o]		

killing again indicates [KIL-sd1] plasmid loss in a *SKI*$^+$ strain, rather than failure of expression. Moreover, if failure of expression of killer-plasmid information were the cause of the K$^-$R$^-$ phenotype, then the *ski2–2 / SKI*$^+$ [KIL-sd1] diploids that had become K$^-$ would yield 2 K^{++} : 2 K$^-$ tetrads instead of the observed 4 K$^-$: 0. The few stable K$^+$ single colonies of the diploid W132 (initially *ski2–2 / SKL*$^+$ [KIL-sd1]) were found to be mitotic recombinants that had become homozygous for the *ski2–2* mutation (data not shown).

In ski2–2 *strains, [KIL-k] mutates to [KIL-sd]*: [KIL-sd1] was discovered as a mutant killer plasmid in a *ski2–2* strain. Another *ski2–2* strain was cured of its killer genome by growth at 37° and of its mitochondrial genome by growth in ethidium bromide. The resulting strain, AT158 = *α ade1 ski2–2* [KIL-o]Δ ρ^0, was used as the recipient in a cytoduction from AT129 = **a** *leu1 kar1–1* [KIL-k] ρ^+ in two separate experiments. In each case, a single K$^+$ ρ^+ *leu*$^+$ *ade*$^-$ single colony was picked, and each was crossed with a *SKI*$^+$ [KIL-o] strain and meiotic tetrads were dissected (crosses W327 and W295, Table 2). In each case, segregation was essentially 2 K^{++} : 2 K$^-$, indicating that the killer genome population had entirely or almost entirely changed to [KIL-sd]. This phenomenon could be explained by either selective replication of occasional [KIL-sd] genomes in a normal population or some form of high-frequency directed mutagenesis of the normal killer genome to the [KIL-sd] form.

[KIL-sd1] can replicate in other ski *mutants*: The replication of [KIL-sd1] depends on the *ski2–2* mutation. To determine whether other *ski* mutations allow [KIL-sd] to replicate, cytoplasm containing [KIL-sd1] was introduced into the strains to be tested, using the *kar1* strain described by CONDE and FINK (1976).

To construct a donor strain, AT129 (**a** *leu1 kar1–1* [KIL-k]) was crossed with W95–13A (*α lys10 ski2–2* K^{++}). Since this diploid contained the wild-type

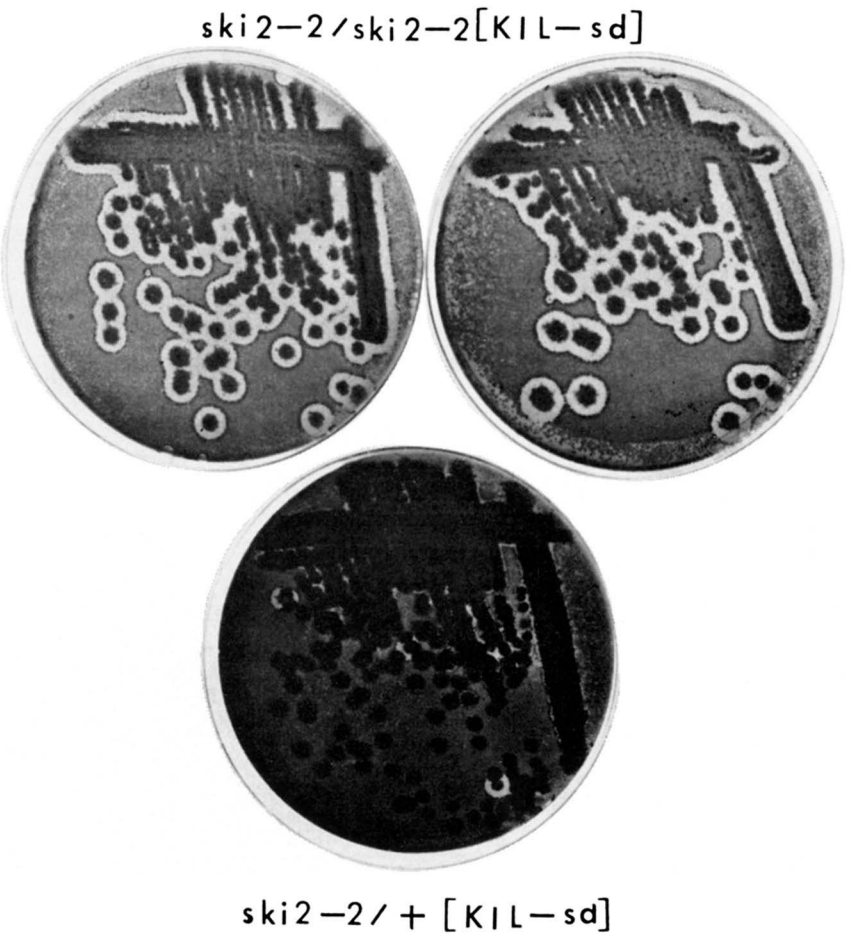

FIGURE 1.—*Ski2*/+ [KIL-sd] heterozygote loses [KIL-sd] plasmid. Diploids obtained by crossing a *ski2–2* [KIL-sd1] strain to *ski2–2* [KIL-sd1] or *ski2–2* [KIL-o]Δ or + [KIL-o] strains were streaked on YPAD. The killer phenotype was visualized by replica-plating colonies on YPAD to MB medium with a sensitive lawn. Top left: *ski2–2* [KIL-sd1] (AT133) × *ski2–2* [KIL-sd1] (AT134). Top right: *ski2–2* [KIL-sd1] (AT133) × *ski2–2* [KIL-o]Δ (AT185). Bottom: *ski2–2* [KIL-sd1] (AT133) × + [KIL-o] (W89-1D).

killer plasmid, all the segregants were K^+ or K^{++}, and the *ski* phenotype segregated 2 : 2. Several *ski2*$^-$ segregants were selected and tested for their ability to transfer cytoplasm to a ρ° *cyh2 KAR*$^+$ tester strain. The *ski2–2 kar1–1* K^+ strain might have [KIL-sd] or [KIL-k], or both. Killer plasmids were eliminated from this strain by heat, and then the [KIL-sd1] plasmid was reintroduced to the cured strain by cytoduction. The genotype of the donor strain (AT197) was thus *ski2–2 kar1–1* [KIL-sd1].

ski 2–3, *ski2–2*, *ski3–3*, *ski 4–1*, *ski1–1*, and SKI^+ strains were cured of both killer and mitochondrial genomes and were used as recipients (Table 4). Strains having nuclear markers of each recipient and having acquired ρ were considered

TABLE 4

[KIL-sd] can replicate in any ski *mutant strain*

	Donor	Recipient	Killing of ρ^+ cytoductants
1st	α *ski2–2* [KIL-sd1] $\rho+$	**a** *kar1 ski2–2* [KIL-o] ρ°	K++ (AT197)*
2nd	**a** *ski2–2 kar1* [KIL-sd1] $\rho+$ (AT197)	α *kar1* ρ° K−	K−
		α *ski2–2* ρ° K−	K++
		α *ski2–3* ρ° K−	K++
		α *ski3–3* ρ° K−	K++
		α *ski4–1* ρ° K−	K++
		α *ski1–1* ρ° K−	K+ (weak)

* Strain AT197 was constructed in the first cytoduction for use as donor in the second series of cytoductions (see text).

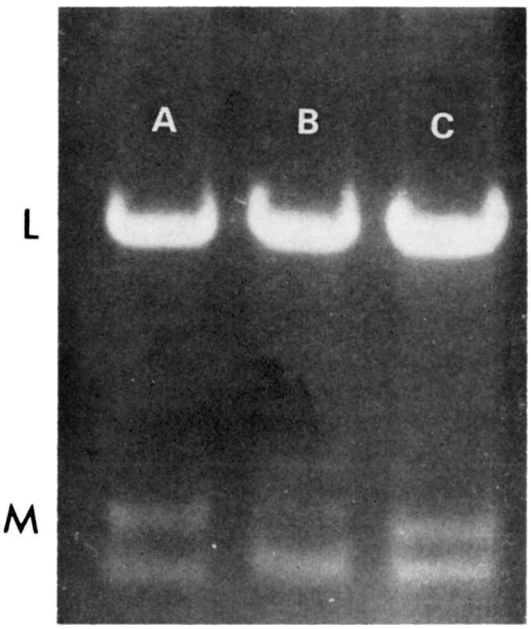

FIGURE 2.—Elimination of [KIL-sd] plasmid from *ski2–2*/+ strain. RNA extraction from intact cells was done by the method described by FRIED (1978). (A) *Ski2–2* [KIL-sd1] (AT133) and + [KIL-kd] (AT255) strains grown separately were mixed together before RNA extraction. The upper band in the M region is [KIL-sd1]; the lower band in the M region is [KIL-kd]. (B) Mixed population of diploids formed by mating diploids *ski2–2* [KIL-sd1] (AT133) and + [KIL-kd] (AT255). The upper band [KIL-sd1] has almost completely disappeared. (C) Mixed population of diploids formed by mating + [KIL-k] (AT159) and + [KIL-kd] (AT255). Both the [KIL-k] (upper band in M region) and [KIL-kd] (lower band) remain at equal intensity.

to be cytoductants, and their killer phenotype was scored. As shown in Table 4, all *ski* mutants so far tested can maintain the [KIL-sd1] plasmid, but wild-type strains cannot. All killer cytoductants except the *ski1–1* strain were superkillers.

Double-stranded RNA analysis: When dsRNA was analyzed by agarose gel electrophoresis, no difference was observed between [KIL-k] and [KIL-sd1]. A killer plasmid mutant, designated [KIL-kd], which is a little shorter than the parental plasmid, was fortuitously found (to be published elsewhere). This plasmid behaves like a wild-type killer plasmid with regard to the killer phenomenon. By using the [KIL-kd] plasmid, specific elimination of the [KIL-sd] plasmid from a *ski2–2 / SKI*$^+$ diploid was demonstrated (Figure 2). Diploids were prepared by crossing a [KIL-kd]-carrying strain with *ski2–2* [KIL-sd1] or *SKI*$^+$ [KIL-k] strains. Double-stranded RNA was extracted from these diploids and analyzed on agarose gel electrophoresis. RNA extracted from mixed cells of [KIL-kd]- and [KIL-sd1]-carrying strains, which were grown separately, was also examined as a control. The result clearly shows that [KIL-sd1] is preferentially eliminated from *ski2–2 / SKI*$^+$ heterozygous diploids. This result implies that the [KIL-sd1] mutation is, in fact, a mutation of the killer plasmid rather than of some other plasmid. Moreover, preferential loss of the [KIL-sd1] plasmid from the *ski2–2 / SKI*$^+$ heterozygotes is probably not due to a suppressive effect by the [KIL-kd] plasmid in these diploids, since [KIL-kd] / [KIL-k] diploids maintain both plasmids (Figure 2C).

DISCUSSION

Several kinds of mutations of the killer plasmid have been described: neutral [KIL-n] (SOMERS and BEVAN 1969), suppressive [KIL-s] (SOMERS 1973), diploid-dependent [KIL-d] (WICKNER 1976), and plasmid mutants designated I$^+$T$^\circ$ and I$^+$T^{sup} (VODKIN, KATTERMAN and FINK 1974). All of these mutant plasmids can replicate in a wild-type background.

A new type of mutant plasmid, designated [KIL-sd], was found in a *ski2–2* strain, and its replication was shown to be dependent on the presence of any one of the *ski* mutations. In each of two independent experiments, when the wild-type [KIL-k] plasmid was introduced into *ski2–2* [KIL-o], the plasmid acquired a [KIL-sd] mutation. Thus, the *ski2–2* mutation might strongly select for [KIL-sd]. Alternatively, it might induce [KIL-sd] mutations. The [KIL-sd] mutation has never been detected in *ski* strains other than *ski2–2* strains, although all *ski* strains tested (including *ski2–3*) allow a [KIL-sd] plasmid to replicate. This allele-specific phenomenon suggests that the *ski2–2* product is directly involved in the conversion from [KIL-k] to [KIL-sd]. However, it remains to be shown, by co-segregation analysis, that the *ski2–2* mutation induces the conversion of [KIL-k] to [KIL-sd]. The determination of the nature of the [KIL-sd] mutation will also be of great interest.

Chromosomal genes (*mak* genes) are necessary to maintain the killer plasmid (WICKNER and LEIBOWITZ 1976; WICKNER 1978), and some *mak* mutants can be bypassed by *ski* mutations; *mak1*, *mak4*, *mak6*, and *mak7* are bypassed by any

one of the *ski* mutations (TOH-E and WICKNER, unpublished). Thus, the [KIL-sd] mutation resembles those *mak* mutations in its defect in plasmid maintenance and in its response to *ski* mutations. This provides the first evidence for a killer genome-coded function essential for its own maintenance or replication. It would be interesting to know whether the *mak*-like phenotype shown by [KIL-sd] mutant is recessive or dominant.

The bypass of the [KIL-sd] mutations by *ski* mutations provides further evidence that the *ski* gene products are involved in killer plasmid replication.

Note added in proof: We have recently found that *ski1–1*, *ski2–3* and *ski4–1* strains initially carrying the wild-type killer plasmid may eventually come to carry only a [KIL-sd] mutant plasmid, though this process is not as rapid or uniform as in the case of *ski2–2* strains.

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