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Intergeneric Transfer of Deoxyribonucleic Acid Killer Plasmids, pGKl1 and pGKl2, from *Kluyveromyces lactis* into *Saccharomyces cerevisiae* by Cell Fusion

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Two novel linear deoxyribonucleic acid plasmids, pGKl1 and pGKl2, were isolated from the yeast *Kluyveromyces lactis*. *K. lactis* strains harboring the pGKl plasmids killed a certain group of yeasts, including *Saccharomyces cerevisiae, Saccharomyces italicus, Saccharomyces rouxii, K. lactis, Kluyveromyces thermotolerans, Kluyvermyces vanudenii, Torulopsis glabrata, Candida utilis,* and *Candida intermedia*. In this experiment, the pGKl1 and pGKl2 plasmids were intergenerically transferred from a *K. lactis* killer strain into a nonkiller (killer-sensitive) strain of *S. cerevisiae* by the use of a protoplast fusion technique. Both of the pGKl plasmids replicated autonomously and stably in the new host cells of *S. cerevisiae* and could coexist with the resident 2-μm deoxyribonucleic acid plasmid. The *S. cerevisiae* cells which accepted the pGKl plasmids expressed the same killer phenotype as that of the donor *K. lactis* killer and became resistant to the *K. lactis* killer. The pGKl plasmids existing in the *S. cerevisiae* cells were cured by treatment with ethidium bromide, and the killer and resistance characters were simultaneously lost. From there results, it was concluded that both the killer and the resistance genes are located on the pGKl plasmids.

A certain group of the yeast *Saccharomyces cerevisiae* is known to secrete a protein toxin (3) and to kill sensitive strains of the same species (31) and other yeasts, such as *Torulopsis glabrata* (4). They are resistant to toxin produced by themselves. The killer and resistance characters are coded for by a double-stranded RNA (dsRNA), M dsRNA, of molecular weight 1.2×10^6 to 1.5×10^6 (26, 29). A species of dsRNA of molecular weight 2.5×10^6 to 3.0×10^6, L dsRNA, is also contained in all killer and in most nonkiller strains as well (2, 24, 26, 30). Both M and L dsRNA's are separately encapsulated in intracellular virus-like particles (18, 23) and are extrachromosomally transmitted during meiosis (30). L dsRNA codes for the capsid protein of virus-like particles (19). Chromosomal genes as well as dsRNA plasmids are responsible for the expression of the killer and resistance phenotypes and also for the maintenance or replication of the plasmids (30, 31), thus providing a suitable model for the understanding of the nucleo-cytoplasmic relationship in eucaryotes. *S. cerevisiae* also harbors a 2-μm DNA plasmid. The number of 2-μm DNA plasmid copies per haploid genome has been estimated to be 50 to 100 (5, 7, 13). By joining with bacterial plasmids, the 2-μm DNA plasmid has been used in genetic manipulation as a shuttle vector between yeast and *Escherichia coli* (1, 11, 25). The biological function of the 2-μm DNA plasmid, however, is not known so far.

Previously (17), we reported the isolation of two linear DNA plasmids, pGKl1 (5.4×10^6) and pGKl2 (8.4×10^6), from the *Kluyveromyces lactis* yeast. They existed extrachromosomally and possessed the same buoyant density of 1.687 g/cm^3, distinguishable from the nuclear (1.699 g/cm^3) and mitochondrial (1.692 g/cm^3) DNAs. *K. lactis* strains harboring the pGKl1 and pGKl2 plasmids secreted trypsin-sensitive killer toxin(s) against a certain group of yeasts, including *S. cerevisiae, Saccharomyces italicus, K. lactis, Kluyveromyces thermotolerans,* and *Kluyveromyces vanudenii*. The killing spectrum and the optimum pH of killing action were different from those of the *S. cerevisiae* killer mediated by M dsRNA and were suspected to be under the control of the pGKl plasmids. This was also supported by the simultaneous loss of the killer character with curing of the pGKl plasmids by ethidium bromide treatment or by heat shock. This paper describes a high-frequency transfer of pGKl1 and pGKl2 plasmids

from a *K. lactis* killer strain into a nonkiller strain of *S. cerevisiae* by the use of a cell fusion technique. The introduced pGKl plasmids replicated and expressed the killer function in the new host of *S. cerevisiae.*

MATERIALS AND METHODS

Strains. The *S. cerevisiae* nonkiller (sensitive) strain was AH22 (**a** *leu2-3, 2-112 his4-519 can1* [KIL-0]). The *K. lactis* killer strain carrying the pGKl1 and pGKl2 plasmids was 2105-1D (α *ade1 ade2 leu*). [KIL-0] here denotes the absence of the M dsRNA plasmid responsible for the killer character of *S. cerevisiae.* The same gene symbols (**a**/α *leu*) were used in the *S. cerevisiae* and *K. lactis* strains, but they belong to different allele system: a strain of *K. lactis* is unable to mate with either **a** or α strains of *S. cerevisiae.* The *leu* genes in both yeasts do not refer to their being alleles.

Media. Complete (YEPD) medium contained 1% yeast extract, 2% peptone, and 2% glucose. Minimal (NBG) medium contained 0.6% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) and 2% glucose. When necessary, NBG medium was supplemented with leucine (50 mg/liter), histidine (50 mg/liter), and adenine sulfate (50 mg/liter). The medium was solidified by the addition of 2% agar, unless otherwise indicated. Canavanine resistance was diagnosed by growth on YEPD agar plates containing canavanine (30 mg/liter).

Assay of killer and resistance characters. The killer and resistance characters were assayed as described by Somers and Bevan (24), using an assay medium (1% yeast extract, 2% peptone, 2% glucose, 3 mg of methylene blue per 100 ml, and 2.5% agar) buffered at pH 5.0 with 0.05 M citrate and phosphate. For examination of the killer phenotype, cells of the strain to be tested were streaked on cells of a tester-sensitive strain which were spread on the assay medium. After 2 days of incubation at 25°C, a killing zone was observed around the streak of killer yeast. Resistance was judged by the absence of the killing zone when cells of a strain to be tested were spread on the assay medium and streaked by a tester-killer strain.

Protoplast formation. Protoplasts were prepared in essentially the same way as reported previously (16). Cells of *S. cerevisiae* and *K. lactis* were separately grown in YEPD medium at 30°C to the late log phase. The harvested cells were washed in water and incubated in 2-mercaptoethanol (0.2%) and EDTA (60 mM) solution at 30°C for 30 min. After the cells were washed with 0.6 M KCl, they were suspended to give a density of 5×10^8 cells per ml in 0.1 citrate phosphate buffer (pH 6.1) containing 0.6 M KCl and 10 mM EDTA. To the cell suspension was added Zymolyase 60,000 (Kirin Brewery Co., Tokyo) (100 μg/ml), and the mixture was incubated with occasional shaking at 30°C for 60 min. Protoplasts were collected by centrifugation at 2,000 rpm for 10 min at 0°C, washed repeatedly with 0.6 M KCl, and resuspended in a citrate phosphate buffer (pH 6.1) containing 0.6 M KCl and 10 mM EDTA.

Protoplast fusion. Protoplasts prepared from 0.2 g of cells (wet) of *K. lactis* 2105-1D and protoplasts prepared from 0.15 g of cells (wet) of *S. cerevisiae* AH22 were mixed and centrifuged at 2,000 rpm for 10 min at 0°C. The pellet was suspended in 5 ml of 33% polyethylene glycol 4,000 containing 0.6 M KCl and 50 mM $CaCl_2$. The suspension was incubated at 30°C for 30 min with occasional shaking and centrifuged at 2,000 rpm for 10 min at 0°C, and the pellet was resuspended in 5 ml of 0.6 M KCl with 50 mM $CaCl_2$. Then 0.1-ml aliquots of the suspension or appropriately diluted suspension were mixed with 10 ml of selective nutrient medium containing 0.6 M KCl and 3% agar which was melted and maintained at 45°C and immediately poured onto agar plates of the same nutrient medium containing 0.6 M KCl. After 6 to 7 days of incubation at 30°C, the colonies which were produced were picked.

Plasmid detection. DNA plasmids were detected by a modification of the procedure by Cameron et al. (5). Protoplasts were prepared from cells of yeast grown in 40 ml of YEPD medium as described above. They were suspended in 4 ml of 0.1 M citrate phosphate buffer (pH 6) containing 0.8 M sorbitol and 10 mM EDTA, and lysed with sodium dodecyl sulfate at a final concentration of 1%. To the solution was added 10 μl of diethylpyrocarbonate, and the mixture was heated to 65°C for 20 min. After cooling, the mixture was centrifuged, and the nucleic acids in the suspension were precipitated with 2 volumes of alcohol. The pellet was collected by centrifugation and dissolved in 1 ml of TES buffer (30 mM Tris-hydrochloride [pH 8.0], 50 mM NaCl, and 5 mM EDTA). RNase A (preheated at 100°C for 5 min) was added at a final concentration of 50 μg/ml, and the mixture was incubated at 37°C for 60 min. A portion of the DNA sample thus obtained was subjected to agarose gel electrophoresis. RNA plasmids were detected by digestion of a portion of the above nucleic acid sample in TES buffer with DNase (20 μg/ml) in place of RNase A, followed by agarose gel electrophoresis.

RESULTS

Transfer of the pGKl plasmids into *S. cerevisiae.* A mixture of protoplasts from *K. lactis* 2105-1D and from *S. cerevisiae* AH22 was treated with polyethylene glycol as described above. When aliquots of the treated samples (at least 5×10^7 protoplasts from each of the parental strains) were plated onto 0.6 M KCl-containing NBG plates and 0.6 M KCl-containing NBG plates supplemented with either leucine or histidine, no colonies were produced. The same results were obtained with repeated experiments, implying that intergeneric nuclear complementation did not occur between the *K. lactis* and *S. cerevisiae* cells. The observation, however, may not rule out the possibility of transfer of cytoplasmic organelles or plasmids from one parent into another. For instance, one could expect a chance transfer of pGKl plasmids from *K. lactis* into *S. cerevisiae*, even in the absence of nuclear fusion or complementation.

To test such a posssibility, the polyethylene

glycol-treated protoplasts were appropriately diluted with 0.6 M KCl containing 50 mM $CaCl_2$ and plated onto 0.6 M KCl-containing NBG plates supplemented with leucine and histidine. The cells of *K. lactis* 2105-1D were unable to grow on this selective medium because of their requirement for adenine, but the protoplasts of *S. cerevisiae* AH22 could regenerate into colonies. As a consequence of the transfer of pGKl plasmids, a portion of regenerated cells of strain AH22 may express the killer phenotype encoded by the plasmids. In two independent experiments, the frequency of colony formation on the selective plates supplemented with leucine and histidine was roughly 1% or less per plate of protoplasts of strain AH22. From these colonies, 243 were picked and tested for the genetic markers. Out of these, 236 showed the same phenotype as that of the parent AH22 (mating type **a**; requirements for leucine and histidine, and resistance to canavanine), and moreover, they were unable to assimilate lactose as a carbon source and were sensitive to cycloheximide (10 μg/ml), indicating that they regenerated from AH22 protoplasts. As described by Lodder (22), *K. lactis* cells could grow on lactate and were resistant to cycloheximide at concentrations at least as high as 200 μg/ml. The remaining seven colonies consisted of a mixture of AH22 and 2105-1D cells. The production of such mixed colonies could be due to mitotic segregation of the parental nuclei from a heterocaryotic state as a result of the fusion between the *K. lactis* and *S. cerevisiae* protoplasts. Alternatively, the protoplasts from *K. lactis* and *S. cerevisiae* may have resulted in mixed aggregates when polyethylene glycol was added, and *K. lactis* cells may have survived on the selective plates by cross-feeding from the surrounding *S. cerevisiae* cells.

The 236 colonies carrying the AH22 phenotype were examined for killing ability towards an *S. cerevisiae* killer strain, B511-4C (ATCC 38659). Out of these, 61 were found to kill B511-4C. This was a characteristic killer phenotype of *K. lactis* strains harboring the pGKl1 and pGKl2 plasmids (17). From the above killer colonies, two colonies, F102-2 and F102-6, were selected, grown for several generations, and replated on YEPD agar for the isolation of single subclones. All of the 120 subclones derived from F102-2 and F102-6 were killers, indicating that the killer phenotype was stably maintained from generation to generation.

Electrophoretic analysis of pGKl plasmids. The above results could be explained by assuming that the transfer of the pGKl plasmids from 2105-1D into AH22 occurred. To confirm this, four subclones derived from killer colony F102-2 (F102-2-1, F102-2-2, F102-2-3, and F102-2-4) and four subclones from the killer colony F102-6 (F102-6-1, F102-6-2, F102-6-3, and F102-6-4) were examined for the existence of pGKl plasmids as described above. Both pGKl1 and pGKl2 plasmids were clearly detected in all of the subclones tested (Fig 1). Figure 1 also indicates the simultaneous existence of a 2-μm DNA plasmid in the host AH22 cells. It thus appears that there is no competitive replication between the plasmids from the two different yeasts, *K. lactis* and *S. cerevisiae*. The killer-sensitive strain AH22 lacked M dsRNA, but contained L dsRNA. All of the subclones isolated from F102-2 and F102-6 also contained L dsRNA, indicating that the introduction of the pGKl plasmids did not affect the maintenance of L dsRNA (data not shown).

Killer and resistance phenotypes encoded by the pGKl plasmids. Previously, we have reported that *K. lactis* strains harboring the pGKl plasmids killed a certain group of yeasts, including *S. cerevisiae* sensitive (G102D and M1-7C [ATCC 38661]) and killer (B060 [ATCC 42750], F38-4A, and B511-4C) strains, *S. italicus* (IFO 0253 and IFO 1049), *K. lactis* (IFO 0433, IFO 1903, L3α, L4, L5, and W600B), *K. thermotolerans* IFO 0662, and *K. vanudenii* IFO 1673 (17). In addition, they were also found to kill *Saccharomyces rouxii* (M1 and M7), *Torulopsis glabrata* (IFO 0005 and IFO 0622), *Candida utilis* IFO 0396, and *Candida intermedia*

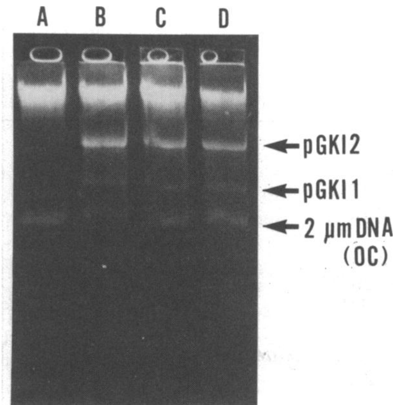

FIG. 1. *Agarose gel electrophoresis of yeast plasmids. Electrophoresis was run from top to bottom. Photograph shows DNA extracts from Saccharomyces cerevisiae AH22 (lane A) and from the fusant S. cerevisiae clones, F102-2-2, F102-2-1, and F102-6-2 (lanes B, C, and D, respectively). The top band of each lane corresponds to nuclear and mitochondrial DNAs. The 2-μm DNA band (OC) indicates the open circular form. The twisted form of the 2-μm DNA plasmid ran off the gel and cannot be seen in the photograph.*

IFO 0761. When the subclones from F102-2 and F102-6 were tested, all of them expressed the same killing spectrum (Table 1). The parent AH22 strain was sensitive to both *K. lactis* killers (IFO 1267 and 2105-1D) and *S. cerevisiae* killers (F38-4A and B511-4C). On examining the subclones from F102-2 and F102-6, they were still sensitive to the *S. cerevisiae* killers, but resistant to the *K. lactis* killers (Table 1). Thus, the pGKl plasmids were proven to encode resistance to *K. lactis* killers as well as the killer phenotype.

Curing of the killer and resistance characters. Cells of F102-2-2 were grown at 30°C in YEPD medium containing ethidium bromide (20 μg/ml) and plated for single colonies. A total of 400 mitochondrial petite colonies were randomly isolated and tested for the killer and resistance phenotypes. Among them, 10 were nonkillers and were sensitive to *K. lactis* killers. Electrophoretic analysis in agarose gel (Fig. 2) indicated that both the pGKl1 and pGKl2 plasmids were lost in these nonkiller sensitive petite strains, providing additional evidence that the pGKl plasmids are responsible for the killer and resistance characters. Of the 10 induced nonkillers, 9 were observed to have the 2-μm DNA plasmid, and 1 did not. This may suggest the simultaneous curing of the 2-μm DNA plasmid with pGKl plasmids by the ethidium bromide treatment. The presence of killer activity in petite strains indicated that the mitochondrial function was not necessary for the expression of the pGKl plasmid-mediated killers, as reported in dsRNA-mediated killers (9).

DISCUSSION

By the use of a protoplast fusion technique, it has become possible to transfer cytoplasmic mitochondria (8, 10) and isolated mitochondria (15, 32) from one yeast cell into another. The fusion technique was also applied to transfer a recombinant DNA plasmid from *E. coli* into *S. cerevisiae* (20). Cells of *Anacystis nidulans* were also incorporated into yeast cells (T. Yamada and K. Sakaguchi, unpublished data).

TABLE 1. *Killing activity of various yeasts*[a]

Streaked strain	Killing of strains: *S. cerevisiae*				*S. italicus*			*S. rouxii*		*K. lactis*						*K. thermotolerans* IFO 0662	*K. vanudenii* IFO 1673	*T. glabrata*		*C. utilis* IFO 0396	*C. intermedia* IFO 0761	Fusant *S. cerevisiae*				
	AH22	G102D	F38-4A*	B511-4C*	IFO 0253	IFO 0725	IFO 1049	M1	M7	IFO 0433	IFO 1903	L3a	L4	L5	W600B			IFO 0005	IFO 0662			F102-2-1**	F102-2-2**	F102-6-1**	F102-6-2**	EtBr-cured F102-2-2
S. cerevisiae																										
AH22	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F38-4A*	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	+
B511-4C*	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	+
K. lactis																										
IFO 1267**	+	+	+	+	±	−	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	−	−	−	−	+
2105-1D**	+	+	+	+	±	−	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	−	−	−	−	+
Fusant *S. cerevisiae*																										
F102-2-1**	+	+	+	+	±	−	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	−	−	−	−	+
F102-2-2**	+	+	+	+	±	−	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	−	−	−	−	+
F102-6-1**	+	+	+	+	±	−	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	−	−	−	−	+
F102-6-2**	+	+	+	+	±	−	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	−	−	−	−	+
EtBr-cured F102-2-2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Assay was done at pH 5 and at 25°C. +, Killing; −, nonkilling; ±, weak killing; *, dsRNA-mediated killer; **, pGKl plasmid-mediated killer. Killing activity was also tested against *K. lactis* strains (IFO 0648, IFO 1090, IFO 1267, K43, WM37, and K51), *K. thermotolerans* strains (IFO 1050, IFO 1674, IFO 1778, IFO 1779, and IFO 1780), *K. africanus* IFO 1671, *K. drosophilarum* IFO 1012, *K. marxianus* IFO 0219, *K. phaffi* IFO 1672, *K. polysporus* IFO 0996, *K. wickerhamii* IFO 1675, and *Schizosaccharomyces pombe* strains (M210, SG55 and M216). All of these strains were insensitive to *K. lactis* killers (IFO 1267 and 2105-1D) (17) and also to the fusant *S. cerevisiae* killer strains (F102-2-1, F102-2-2, F102-6-1, and F102-6-2).

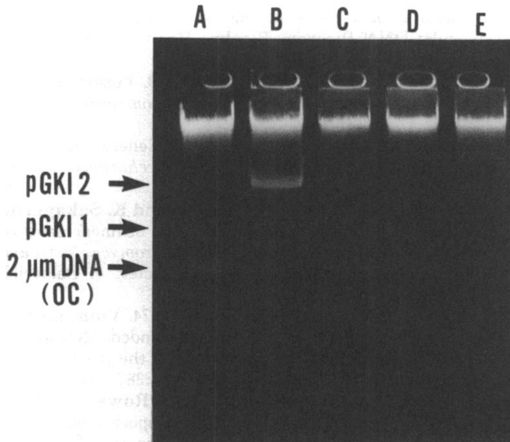

FIG. 2. *Electrophoresis of DNA extracts from ethidium bromide-induced nonkiller strains. Lane A, DNA extract from S. cerevisiae AH22; lanes B, C, D, and E, DNA extract from a fusant clone F102-2-2 and the ethidium bromide-cured clones no. 1, no. 2 and no. 3, respectively. Cured clone no. 3 was also devoid of the 2-µm DNA plasmid.*

In this experiment, the transfer of the pGKl plasmids from *K. lactis* into *S. cerevisiae* was attempted. Taxonomically, the *K. lactis* yeast is unable to mate with *S. cerevisiae*, and the intergeneric nuclear complementation was not observed in the protoplast fusion. Under these conditions, it was surprising that the pGKl plasmids were transferred from *K. lactis* 2105-1D into *S. cerevisiae* AH22 with an unexpectedly high frequency by protoplast fusion. In fact, nearly a quarter of the colonies regenerated from *S. cerevisiae* protoplasts contained both the pGKl1 and pGKl2 plasmids. The cells of AH22 strain which accepted the pGKl plasmids expressed the same killer phenotype as that of the *K. lactis* killer strain 2105-1D. After the introduction of the pGKl plasmids into the AH22 strain, the *S. cerevisiae* cells became resistant to *K. lactis* killers but were still sensitive to *S. cerevisiae* killers (F38-4A and B511-4C), indicating that the mechanism of killer resistance is different between the two killer yeast systems mediated by the pGKl and dsRNA plasmids, respectively. This is in agreement with the fact that all *K. lactis* strains, regardless of the presence or absence of pGKl plasmids, were insensitive to *S. cerevisiae* killers mediated by the dsRNA plasmid (17). Except for the killer and resistance characters, the AH22 cells transferred with the pGKl plasmids were completely devoid of genetic and taxonomic traits from the *K. lactis* strain 2105-1D; (i) they possessed the same nuclear genotype (*a leu2-3,2-112 his4-519 can1*) as that of AH22, (ii), they were unable to mate with *K. lactis* strains of either mating type **a** or α, (iii) they showed morphological characteristics of *S. cerevisiae*, (iv) they, as well as AH22, were unable to grow on YEPD medium containing cycloheximide (10 µg/ml), whereas *K. lactis* cells were resistant to cycloheximide (at least 200 µg/ml) (22), and (v) they were unable to assimilate lactose as a carbon source, unlike *K. lactis* cells (22). Thus, the ability of the pGKl plasmids to replicate and function in *S. cerevisiae* was clearly demonstrated.

That the killer and resistance characters are under the control of the pGKl plasmids was proven not only by the cell fusion, but also by the curing experiment with ethidium bromide. In a different line of experiments, the simultaneous curing of pGKl1 and pGKl2 plasmids from *K. lactis* killers was also observed by UV irradiation or by heat shock, accompanied by the loss of the killer and resistance characters. Some *K. lactis* nonkiller derivatives were observed to be devoid of only the pGKl1 plasmid or to carry an alternative new plasmid with a reduced molecular weight compared with the pGKl1 plasmid, whereas the pGKl2 plasmid was still maintained (O. Niwa, K. Sakaguchi and N. Gunge, manuscript in preparation). This bears a marked analogy to certain nonkiller derivatives of the *S. cerevisiae* killer which were characterized by the loss or deletion of M dsRNA (26–28, 30). In the course of the curing experiments, it was also found that the efficiency of curing varied, depending on the strains employed. This could explain why it was so difficult to cure the pGKl plasmids from a *K. lactis* killer strain in our previous work (17).

Spontaneous loss of the 2-µm DNA plasmid in *S. cerevisiae* was reported (14, 21), but curing with ethidium bromide has so far been unsuccessful (6, 12). In the present study, the curing of pGKl plasmids was observed by growing cells of a fusant *S. cerevisiae* killer strain in the presence of ethidium bromide. One of the 10 cured clones also lacked the resident 2-µm DNA plasmid. The loss of the 2-µm DNA plasmid may have occurred spontaneously, but it is tempting to assume that it was induced by ethidium bromide. It is conceivable that the detection of the curing of the 2-µm DNA plasmid has been difficult because of the unknown function. In comparison, the loss of the coexisting killer plasmids could be easily recognized, and this may have facilitated detection.

In view of a highly efficient incorporation of the pGKl plasmids into *S. cerevisiae*, it may be also possible to transfer the pGKl plasmids into various yeast species or other eucaryotes by cell fusion or by transformation with isolated pGKl plasmids. Such attempts are now in progress.

The finding of the intergeneric transfer of the pGKl plasmids associated with the killer character will be useful in providing a powerful tool in the genetic manipulation and elucidation of the genetics and the molecular biology of yeasts.

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