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Isolation and Characterization of Linear Deoxyribonucleic Acid Plasmids from *Kluyveromyces lactis* and the Plasmid-Associated Killer Character

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Two linear deoxyribonucleic acid plasmids, designated pGKl1 and pGKl2, were isolated from the yeast *Kluyveromyces lactis* IFO 1267. pGKl1 and pGKl2 had molecular weights of 5.4×10^6 and 8.4×10^6, respectively. Both plasmids possessed the same density of 1.687 g/cm^3, lighter than the densities of mitochondrial (1.692 g/cm^3) and nuclear (1.699 g/cm^3) deoxyribonucleic acids. A restriction map of pGKl1 was constructed from digestions by *Eco*RI, *Hin*dIII, *Pst*I, and *Bam*HI. pGKl2 was cleaved by *Eco*RI into seven fragments and by *Bam*HI into two fragments. *K. lactis* IFO 1267 killed *Saccharomyces cerevisiae* sensitive and killer strains and certain strains of *Saccharomyces italicus*, *K. lactis*, *Kluyveromyces thermotolerans*, and *K. vanudenii*. All *K. lactis* strains lacking the pGKl plasmids were nonkillers. A hybrid was constructed between *K. lactis* IFO 1267 and a nonkiller *K. lactis* strain lacking the plasmids and subjected to tetrad analysis after sporulation. The killer character was extrachromosomally transmitted in all tetrads in association with the pGKl plasmids. The double-stranded ribonucleic acid killer plasmid could not be detected in any *K. lactis* killer strains. It is thus highly probable that the killer character is mediated by the linear deoxyribonucleic acid plasmids. A single chromosomal gene was found which was responsible for the resistance to the *K. lactis* killer.

The presence of a 2-μm circular DNA plasmid in the yeast *Saccharomyces cerevisiae* has been reported previously (4, 11, 15, 27). Attempts to isolate 2-μm DNA from nuclei or mitochondria were unsuccessful (2, 5). Recently, however, the association of 2-μm DNA with folded chromosomes has been reported (32). The replication or maintenance is under the control of nuclear genes, whereas the mode of inheritance is non-Mendelian (18, 19). The 2-μm DNA was joined with bacterial plasmids, and the resulting hybrid DNA molecules were used as shuttle vectors for genetic manipulation in yeast and *Escherichia coli* systems (1, 6, 9, 30). The existence of 2-μm or 2-μm-like DNA in *Saccharomyces italicus* (16) and *Schizosaccharomyces pombe* (6) has also been demonstrated; however, no other DNA plasmids have been detected in yeasts. We have searched for plasmids among 70 strains of yeasts belonging to 17 different genera. As a result, a strain of the petite negative yeast *Kluyveromyces lactis* was found to harbor two novel linear DNA plasmids, designated pGKl1 and pGKl2. The *K. lactis* strain with the pGKl plasmids expressed a killer phenotype toward all of the *Saccharomyces cerevisiae* strains tested and certain strains of *S. italicus* and *Kluyveromyces* spp. The present paper describes the isolation and characterization of the linear DNA plasmids from yeasts and the possible control of the killer character by the plasmids.

MATERIALS AND METHODS

Strains used for the plasmid detection. The following species (and strains) were used: *Saccharomyces cerevisiae* (G706, 1035-186, O708-11-16A, F38-4A, B060 AF-1, 3026-1, 3027-1, KL-88, 55R5-3C/1, and H-1), *Saccharomyces italicus* (IFO 0253, IFO 0725, and IFO 1049), *Saccharomyces rouxii* (NISL-A31), *Schizosaccharomyces pombe* (IFO 0346, M210, M216, and SG55), *Saccharomycodes ludwigii* (M10, M15, M18, M35, and M38), *Candida krusei* (IFO 0839 and IFO 1395) *Candida lipolytica* (NRRL-Y-6795), *Candida solani* (MCI 0632), *Candida tropicalis* (IFO 1400), *Torulopsis famata* (RIFY-7455), *Torulopsis candida* (IFO 0664), *Citeromyces matritensis* (IFO 0954), *Pachysolen tannophilus* (IFO 1007), *Debaryomyces hansenii* (IFO 0855), *Metschunikowia pulcherima* (IFO 1407 and IFO 1405), *Metschunikowia bicuspidata* (MCI 1027), *Pichia membranefaciens* (IFO 0864), *Rhodosporidium toruloides* (IFO 8766), *Leucosporidium scotti* (IFO 1212 and MCI 1026), *Kluyveromyces lactis* (IFO 0433, IFO 0648, IFO 1090, IFO 1267, IFO 1903, WM37, W600B, L3α, K43, L4, K5, and K51), *Kluyveromyces fragilis* (ATCC 10022), *Kluyveromyces thermotolerans* (IFO 0662, IFO 1050, IFO 1674, IFO 1778, IFO 1779, and IFO 1780), *Kluyveromyces drosophilarum* (IFO 1012), *Kluyveromyces*

marxianus (IFO 0219), *Kluyveromyces africanus* (IFO 1671), *Kluyveromyces phaffi* (IFO 1672), *Kluyveromyces vanudenii* (IFO 1673), *Kluyveromyces wickerhamii* (IFO 1675), *Botryoascus synaedendrus* (MCI 1025), *Sporidiobolus johnsonii* (MCI 1030), *Endomycopsis fibuligera* (MCI 2200), *Hansenula anomala* (MCI 2321), and *Hansenula capsulata* (MCI 2325).

Detection and preparation of plasmid DNA. Plasmid DNA was detected by a modification of the procedure of Hirt (14). Cells of yeast grown in 40 ml of YEPD medium (2% peptone, 1% yeast extract, 2% glucose) were converted to protoplasts with Zymolyase 60,000 as described previously (12). The protoplasts were washed and suspended in 4 ml of 0.1 M citrate-phosphate buffer (pH 6) containing 0.8 M sorbitol and 0.01 M EDTA and then lysed by adding 0.5 ml of 10% sodium dodecyl sulfate solution. After the addition of 5 M NaCl at a final concentration of 1 M, the mixture was kept on ice overnight and subjected to centrifugation at 17,000 rpm for 30 min. To the supernatant were added two volumes of cold (−20°C) ethanol and one-tenth volume of 3 M sodium acetate. The mixture was incubated at −20°C for 2 h. The precipitate was collected by centrifugation at 17,000 rpm for 20 min and dissolved in 100 μl of TES buffer (30 mM Tris-hydrochloride [pH 8.0], 50 mM NaCl, 5 mM EDTA) containing 0.4% Sarkosyl. RNase A (Sigma Chemical Co.; preheated for 5 min at 100°C) was added at a final concentration of 50 μg/ml, and the mixture was incubated for 30 min at 37°C. A portion of the DNA sample thus obtained was subjected to agarose gel electrophoresis. Plasmid DNA bands were visualized under UV (365-nm) light.

Plasmid DNA was separated from gels by the freeze and squeeze method described by Tanaka and Weisblum (33), and the recovered DNA was purified by removing ethidium bromide with butanol treatment and dialyzing against 0.1× SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 1 mM EDTA.

Detection of double-stranded RNA (dsRNA) plasmids. Nucleic acid fraction was collected from the sodium dodecyl sulfate lysate of yeast protoplasts by adding two volumes of cold ethanol as described in detection of plasmid DNA. A portion of the sample was digested with DNase (20 μg/ml) and subjected to 0.7% agarose gel electrophoresis for the detection of RNA plasmids. Cytoplasmic polyhedrosis virus RNA, which consists of 10 dsRNA segments of known molecular weights (8), was used for molecular weight markers.

Digestion with restriction endonucleases. *Eco*RI, *Hin*dIII, *Bam*HI, and *Pst*I were purchased from Boehringer Co., and *Hpa*I was purchased from Bethesda Research Laboratories, Inc. Digestions were carried out for 1 h at 37°C in the following buffers: *Eco*RI buffer (10 mM $MgCl_2$, 50 mM NaCl, 100 mM Tris-hydrochloride at pH 7.5), *Hin*dIII buffer (7 mM $MgCl_2$, 60 mM NaCl, 7 mM Tris-hydrochloride at pH 7.4), *Bam*HI buffer (10 mM $MgCl_2$, 100 mM Tris-hydrochloride at pH 7.5), *Pst*I buffer (10 mM $MgSO_4$, 90 mM Tris-hydrochloride at pH 7.5), and *Hpa*I buffer (20 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 10 mM Tris-hydrochloride at pH 7.4). Double digestions were done with *Eco*RI plus *Hin*dIII in *Hin*dIII buffer, with *Eco*RI plus *Pst*I in *Eco*RI buffer, with *Hin*dIII plus *Pst*I in *Hin*dIII buffer, and with *Pst*I plus *Bam*HI in *Bam*HI buffer. Double digestions with *Eco*RI plus *Bam*HI and with *Bam*HI plus *Hin*dIII were first done with *Bam*HI in *Bam*HI buffer and then redigested after adjusting the NaCl concentration to 50 mM with *Eco*RI and *Hin*dIII, respectively. The reactions were terminated by the addition of BJ solution (60% sucrose, 0.005% bromophenol blue, 0.08 M Tris, 0.04 M sodium acetate, 0.002 M sodium EDTA, and 0.5 μg of ethidium bromide per ml at pH 7.9) and heated for 5 min at 65°C before agarose gel electrophoresis. For determination of molecular weights of digested DNA, *Hin*dIII fragments of λ DNA and *Hin*cII fragments of φX174 DNA were used as molecular weight markers.

Test of killer phenotypes. The killer phenotype was assayed by the methylene blue agar technique described by Somers and Bevan (29). Cells of tester strains grown in YEPD medium were suspended in sterile water; 0.2 ml of the suspension (10^7 cells per ml) was spread on an assay medium (1% yeast extract, 2% peptone, 2% glucose, 3 mg of methylene blue per 100 ml, and 2.5 to 3.5% agar) adjusted to pH 4.5, 5.0, 6.0, and 7.0 with 0.05 M citrate-phosphate buffer. For examination of the killing activity, cells of various yeast strains grown on YEPD agar were streaked onto the above assay medium on which cells of a tester strain were spread. After 2 to 3 days of incubation at 25°C, a clear killing zone was observed around the streaks of killer yeasts. The following *S. cerevisiae* strains were used as tester strains: killer strains BO60 (**a** haploid), F38-4A (α haploid), and B511-4C (α/α diploid) and sensitive strains G102D (α haploid) and M1-7C (α/α diploid).

Genetic analysis. Tetrad analysis was conducted with the aid of a de Fonbrune micromanipulator by the procedure of Johnston and Mortimer (17).

RESULTS

Detection of plasmid DNA. Sodium dodecyl sulfate lysates of protoplasts from various yeasts were examined for the existence of plasmids by agarose gel electrophoresis. In investigating 70 strains of yeasts belonging to 33 different species of 17 genera listed above, 2-μm DNA was found in all of 10 *S. cerevisiae* strains, except for strain KL-88, and in two *S. italicus* strains (IFO 0253 and IFO 1049); two novel-type plasmids were found in *K. lactis* IFO 1267. No DNA plasmids were detected among the remaining yeasts.

Figure 1 shows the result of electrophoretic analysis of plasmid samples from *K. lactis* IFO 1267 (lane A), *S. cerevisiae* 0708-11-16A (lane B) and *S. italicus* IFO 0253 (lane C). In lane A, two discrete plasmid bands (pGKl1 and pGKl2) with different mobilities are revealed, in addition to the top band DNAs corresponding to nuclear and mitochondrial DNAs. All of these bands disappeared when treated with DNase. The plasmid and top band fractions from *K. lactis* IFO 1267 were separately isolated from a num-

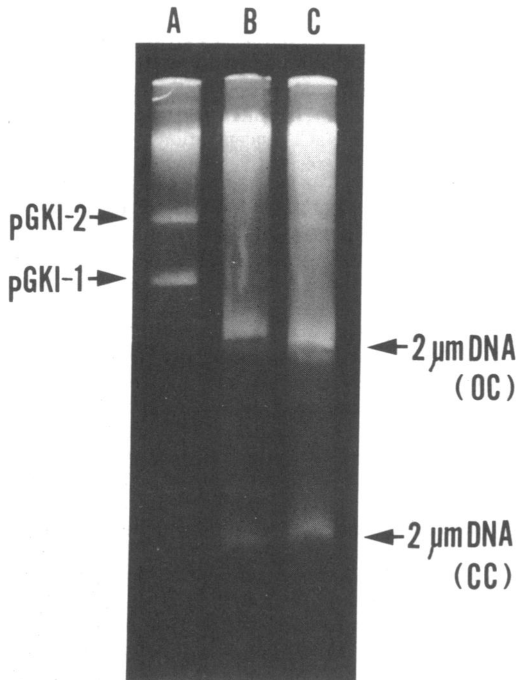

FIG. 1. *Agarose gel electrophoresis of DNA extracts. Electrophoresis is from top to bottom. Photograph shows DNA extracts from K. lactis IFO 1267 (lane A), S. cerevisiae O708-11-16A (lane B) and S. italicus IFO 0253 (lane C). The top band of each lane corresponds to nuclear and mitochondrial DNAs. The 2-μm DNA bands represent the open circular (OC) and closed circular (CC) forms. The bands below the 2-μm DNA (OC) on the B and C lanes are 2-μm DNA dimers (CC), and the faint bands at the position of pGKl2 are the OC form of 2-μm DNA dimers (25).*

ber of gels by the freeze and squeeze technique (33) for further study.

Structure and size of pGKl1 and pGKl2 plasmids. In electron microscope analysis (Fig. 2), the pGKl1 and pGKl2 plasmid fractions purified from gels were found to consist of homogeneous populations of linear DNA of 2.6 to 2.7 μm (molecular weight, 5.4×10^6) and 4.1 to 4.2 μm (molecular weight, 8.4×10^6) in length, respectively. No circular DNA molecules were detected, except for the marker pBR322. Because of the linear structure of the DNA molecules, the estimation of molecular weights based on the mobility by electrophoresis was conducted by comparison with linear fragments from *Hin*dIII digestion of λ DNA. As a result, the pGKl1 and pGKl2 DNAs were shown to have molecular weights of 5.3×10^6 to 5.4×10^6 and 8.3×10^6 to 8.4×10^6, respectively. The values are in agreement with the sizes estimated by electron microscope, evidence of the linear structures of the pGKl plasmids.

Digestion with restriction endonucleases. The pGKl1 and pGKl2 plasmids were treated with *Eco*RI, *Hin*dIII, *Bam*HI, *Pst*I, and *Hpa*I, and the sizes of the resulting fragments were determined by coelectrophoresis with *Hin*dIII digests of λ DNA and *Hin*cII digests of φX174 DNA. The results of single and double digestions of pGKl1 (Table 1) led to the construction of restriction map (Fig. 3). No digestion was observed with *Hpa*I. pGKl2 was digested by *Eco*RI into seven fragments (molecular weights, 2.5×10^6, 2.3×10^6, 0.95×10^6, 0.85×10^6, 0.7×10^6, 0.55×10^6, and 0.5×10^6) and digested by *Bam*HI into two fragments (molecular weights, 4.8×10^6 and 3.6×10^6). Double digestion by *Eco*RI and *Bam*HI indicated that the *Eco*RI fragment (molecular weight, 0.85×10^6) contained the single *Bam*HI site and was digested by *Bam*HI into fragments with molecular weights of 0.65×10^6 and 0.2×10^6. *Hin*dIII, *Pst*I, and *Hpa*I did not cleave pGKl2; therefore, the physical map of pGKl2 was difficult to construct. The existence of several definite cleavage sites for restriction enzymes ruled out the possibility that the linear DNAs may have come from random fragmentations of mitochondrial or chromosomal DNAs during DNA isolation. The top band DNAs, mainly consisting of chromosomal DNA, gave diffuse fragments when digested by *Eco*RI or *Hin*dIII.

Buoyant density. Buoyant densities of the plasmid DNAs were determined in neutral CsCl solution at 40,000 rpm for 42 h at 16°C in a Hitachi analytical centrifuge model 282 by using internal density markers of phage SPO1 DNA ($\rho = 1.742$ g/cm^3) and *E. coli* DNA ($\rho = 1.710$ g/cm^3). Both pGKl1 and pGKl2 plasmids showed an identical density of 1.687 g/cm^3. The DNAs isolated from the top band fraction of gels were

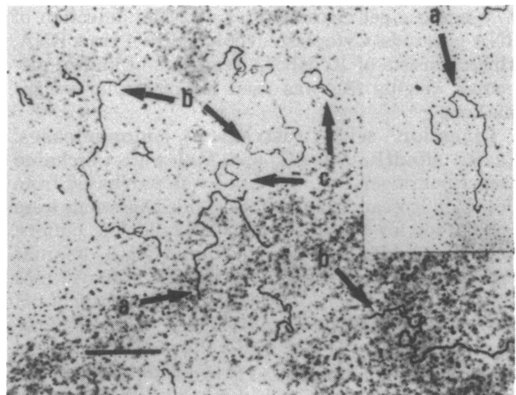

FIG. 2. *Electron micrograph of pGKl1 and pGKl2 plasmids: a, pGKl1; b, pGKl2; c, open circular form of pBR322. The bar represents 1 μm.*

TABLE 1. *Molecular weights of restriction fragments of plasmid pGKl1*

Fragments[a]	Mol wt (10^6) of restriction fragments of the following enzymes:									
	*Eco*RI	*Hin*dIII	*Bam*HI	*Pst*I	*Eco*RI plus *Hin*dIII	*Eco*RI plus *Bam*HI	*Eco*RI plus *Pst*I	*Hin*dIII plus *Bam*HI	*Hin*dIII plus *Pst*I	*Pst*I plus *Bam*HI
A	3.0	3.4	2.7 2.7	3.9	1.8	2.7	2.5	2.1	2.9	2.2
B	2.4	1.5	—[b]	1.0	1.6	2.4	1.4	1.4	1.0	1.7
C	—	0.5	—	0.5	1.5	0.3	1.0	1.3	0.5 0.5 0.5	1.0
D	—	—	—	—	0.5	—	0.5	0.5	—	0.5
Total	5.4	5.4	5.4	5.4	5.4	5.4	5.4	5.3	5.4	5.4

[a] Fragments were designated A to D in order of increasing molecular weight.
[b] —, No fragment.

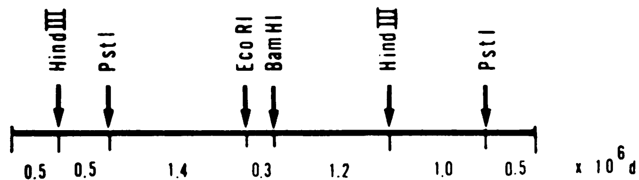

FIG. 3. *Restriction map of the pGKl1 plasmid. The map was constructed on the basis of the data of Table 1.*

resolved into two peaks: a main peak of a density of 1.699 g/cm^3 due to nuclear DNA (20, 21, 28) and a faint peak of a density of 1.692 g/cm^3 due to mitochondrial DNA (20, 21, 28). Thus, the densities of the plasmids are lower than those of the mitochondrial and nuclear DNAs, in contrast to the density of the 2-μm DNA of *S. cerevisiae*, which is the same as the density of nuclear DNA (ρ = 1.700 g/cm^3) and is higher than that of mitochondrial DNA (ρ = 1.683 g/cm^3).

Killing activity of *K. lactis* IFO 1267. Killing activity of yeasts was examined as described above. *S. cerevisiae* killer strains B060, F38-4A, and B511-4C killed *S. cerevisiae* sensitive strains G102D and M1-7C at a suitable pH (pH 5) (Table 2). The killer strains did not kill each other, indicating that they were immune to toxic substances produced by themselves. The 12 *Kluyveromyces lactis* strains listed above were tested for killing activity against *S. cerevisiae* strains and among themselves. As a result, *K. lactis* IFO 1267 carrying the pGKl1 and pGKl2 plasmids was found to kill the *S. cerevisiae* sensitive and killer strains and *K. lactis* strains IFO 1903, IFO 0433, L3α, W600B, L4, and L5. *K. lactis* strains K43, WM37, K51, IFO 0648, and IFO 1090 were not killed. The 11 *K. lactis* strains lacking the pGKl plasmids did not kill any of the strains of *S. cerevisiae* and *K. lactis* mentioned above. All *K. lactis* strains were resistant to the *S. cerevisiae* killers.

To further characterize the killer phenotypes of *K. lactis* IFO 1267, the killing tests were extended to strains of other yeasts: *Saccharomyces italicus*, several *Kluyveromyces* species, and *Schizosaccharomyces pombe* (Table 2). The *S. cerevisiae* killers killed all of the *S. italicus* strains tested, whereas *K. lactis* strain IFO 1267 weakly killed *S. italicus* strains IFO 0253 and IFO 1049, but did not kill *S. italicus* IFO 0725. On the other hand, *K. lactis* IFO 1267 killed *K. thermotolerans* IFO 0622 and *K. vanudenii* IFO 1673, although the *S. cerevisiae* killers and the 11 *K. lactis* strains lacking the pGKl plasmids did not kill them. *Schizosaccharomyces pombe* strains were not killed by either group of yeasts.

The effect of pH on the killing was tested by using *S. cerevisiae* strains as testers. *S. cerevisiae* killers were active in the range of pH 4.5 to 5, but not at pH 6, in agreement with the previous reports (23, 29) (Table 3). In contrast, the killing of *K. lactis* IFO 1267 was expressed in the range of pH 5 to 7, but not at pH 4.5. Thus, the

TABLE 2. *Killing activity of various yeasts*[a]

Streaked strain	Killing of background lawn strains:																																						
	S. cerevisiae					*S. italicus*			*K. lactis*																*K. thermotolerans*														
	G102D	M1-7C	B060	F38-4A	B511-4C	IFO 0253	IFO 0725	IFO 1049	IFO 1903	K43	L3α	WM37	W600B	K51	L4	L5	IFO 0433	IFO 0648	IFO 1090	IFO 1267	Tetrad 8A	Tetrad 8B	Tetrad 8C	Tetrad 8D	IFO 0662	IFO 1050	IFO 1674	IFO 1778	IFO 1779	IFO 1780	*K. africanus* IFO 1671	*K. drosophilarum* IFO 1012	*K. marxianus* IFO 0219	*K. phaffi* IFO 1672	*K. polysporus* IFO 0996	*K. vanudenii* IFO 1673	*K. wickerhamii* IFO 1675	*Schizosaccharomyces pombe* M210	*Schizosaccharomyces pombe* SG55	*Schizosaccharomyces pombe* M216
S. cerevisiae																																							
G102D	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B060	+	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B511-4C	+	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
K. lactis																																							
WM37	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
W600B	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IFO 1267	+	+	+	+	+	±	−	±	+	−	+	−	±	−	+	+	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Tetrad 8A	+	+	+	+	+	±	−	±	+	−	+	−	±	−	+	+	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Tetrad 8B	+	+	+	+	+	±	−	±	+	−	+	−	±	−	+	+	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Tetrad 8C	+	+	+	+	+	±	−	±	+	−	+	−	±	−	+	+	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Tetrad 8D	+	+	+	+	+	±	−	±	+	−	+	−	±	−	+	+	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−

[a] Assays were carried out at pH 5. +, Killing; −, nonkilling; ±, weak killing.

TABLE 3. *Killing action at various pH's*

Streaked strain	Killing of *S. cerevisiae* strains[a]: G102D				B511-4C			
	4.5[b]	5	6	7	4.5	5	6	7
S. cerevisiae								
G102D	−	−	−	−	−	−	−	−
B060	+	+	−	−	−	−	−	−
B511-45C	+	+	−	−	−	−	−	−
K. lactis								
W600B	−	−	−	−	−	−	−	NT
IFO 1267	−	+	+	+	−	+	+	NT
Tetrad 8A	−	+	+	+	−	+	+	NT
Tetrad 8B	−	+	+	+	−	+	+	NT
Tetrad 8C	−	+	+	+	−	+	+	NT
Tetrad 8D	−	+	+	+	−	+	+	NT

[a] NT, Not tested; +, killing; −, nonkilling; ±, weak killing.
[b] pH value.

killer phenotype of *K. lactis* IFO 1267 was distinguishable from that of *S. cerevisiae* with regard to the killing spectrum against various yeasts and the pH range of killing.

Cells of *K. lactis* IFO 1267 were grown in YEPD medium which contained 20% glycerol to stabilize the toxin (22) and centrifuged. The cell-free solution showed the killing activity, but did not when treated with papain.

Association of the killer character with the pGKl plasmids. To understand the genetic relationship of the killer character with the pGKl plasmids, *K. lactis* IFO 1267 (mating type a prototroph) was crossed with *K. lactis* W600B (*α ade1 ade2 leu*), one of the nonkiller *K. lactis* strains lacking the pGKl plasmids, and the resulting hybrid was subjected to tetrad analysis after sporulation. Out of 17 tetrads dissected, all gave 2:2 segregation for mating types and *leu* marker and irregular (2:2, 1:3, and 0:4) segregation for *ade* marker, which was due to the independent segregation of *ade1* and *ade2* mutations involved in the cross. In contrast, the killer phenotype of *K. lactis* IFO 1267 was segregated in a 4:0 ratio (Table 4): all tetrads from the cross killed *S. cerevisiae* sensitive and killer strains, the six nonkiller *K. lactis* strains (IFO 1093, IFO 0433, L3α, W600B, L4, and L5), *S. italicus* IFO 0253 and IFO 1049, *K. thermotolerans* IFO 0622, and *K. vanudenii* IFO 1673, as did *K. lactis* IFO 1267 (Table 2, tetrads 8A to 8D). Again, killing was expressed in the range of pH 5 to 7, but not at pH 4.5, against *S. cerevisiae* strains (Table 3, tetrads 8A to 8D). From these 17 tetrads, 7 were selected at random and examined for the existence of plasmids. All four spores from the tetrads contained both pGKl1 and pGKl2 plasmids (Table 4). Thus, both the pGKl1 and pGKl2 plasmids exhibited an extrachromosomal inheritance and were associated with the killer character during meiosis.

TABLE 4. *Tetrad analysis of a cross between K. lactis IFO 1267 (a) and K. lactis W600B (α ade1 ade2 leu)*[a]

Strain/tetrad	MT	*ade*	*leu*	Killing activity	pGKl1	pGKl2
K. lactis IFO 1267	a	+	+	−	−	−
K. lactis W600B	α	−	−	−	−	−
Tetrad						
1A	a	−	−	+	+	+
1B	α	+	+	+	+	+
1C	a	+	+	+	+	+
1D	α	−	−	+	+	+
3A	α	−	−	+	+	+
3B	α	−	+	+	+	+
3C	a	+	+	+	+	+
3D	a	−	−	+	+	+
5A	a	−	−	+	+	+
5B	a	+	+	+	+	+
5C	α	−	−	+	+	+
5D	α	−	+	+	+	+
8A	a	+	−	+	+	+
8B	a	+	+	+	+	+
8C	α	−	+	+	+	+
8D	α	−	−	+	+	+
14A	α	−	+	+	+	+
14B	α	−	+	+	+	+
14C	a	−	−	+	+	+
14D	a	+	−	+	+	+
16A	a	−	−	+	+	+
16B	a	+	+	+	+	+
16C	α	−	−	+	+	+
16D	α	+	+	+	+	+
18A	α	−	−	+	+	+
18B	a	+	−	+	+	+
18C	α	−	+	+	+	+
18D	a	−	+	+	+	+

[a] Out of 17 tetrads dissected, 7 were selected for the detection of plasmids and the results listed above. The remaining 10 tetrads gave 2:2 segregation for mating types (MT) and *leu* marker and irregular segregation (2:2 in 5, 1:3 in 4 and 0:4 in 1) for *ade* marker. Killing activity segregated at a ratio of 4:0.

Examination of dsRNA plasmids. RNA fractions prepared from protoplast lysate were subjected to agarose gel electrophoresis. As shown in Fig. 4 (lane L), two discrete RNA bands were detected from a killer strain of *S. cerevisiae*: the molecular weights of the upper

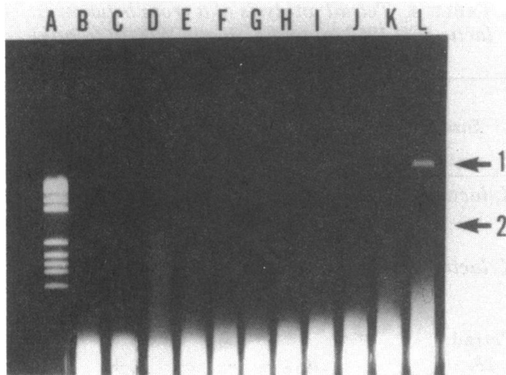

FIG. 4. *Electrophoresis of DNase-treated samples of nucleic acid extract from protoplast lysates. A, cytoplasmic polyhedrosis virus RNA: B, K. lactis IFO 1267; C, K. lactis W600B; D to G, tetrads 3A to 3D, respectively; H to K, tetrads 8A to 8D, respectively; L, S. cerevisiae killer strain F38-4A. Arrows 1 and 2 in lane L correspond to dsRNA's of molecular weights 2.7×10^6 and 1.3×10^6, respectively. Cytoplasmic polyhedrosis virus RNA consists of 10 segments having the following molecular weights ($\times 10^6$): 2.55, 2.42, 2.32, 2.03, 1.82, 1.12, 0.84, 0.62, 0.56 and 0.35 (8).*

and lower bands were estimated to be 2.7×10^6 and 1.3×10^6, respectively, by using cytoplasmic polyhedrosis virus RNA for internal molecular weight markers. The RNA bands were highly resistant to digestion with T_2 RNase at 27°C for 15 min as compared with *E. coli* tRNA (Sigma) (data not shown), indicating that they consisted of dsRNA (26). The RNA fraction obtained from sensitive strains of *S. cerevisiae* gave only a single band corresponding to dsRNA of 2.7×10^6; the dsRNA with a molecular weight of 1.3×10^6 was not detected (data not shown). These data are in agreement with the results of killer and sensitive strains of *S. cerevisiae* reported previously (2, 31, 35). When *K. lactis* strains IFO 1267 and W600B and tetrads from the cross were examined by the same procedure, no dsRNA bands could be detected (Fig. 4, lanes B to K). Similarly, all of the 11 nonkiller strains of *K. lactis* lacked both dsRNA's. Thus, the killer character of *K. lactis* strains could not be due to the dsRNA.

A chromosomal gene controlling the resistance to the *K. lactis* killer. *K. lactis* killer strains (*K. lactis* IFO 1267 and tetrads from the cross with strain W600B carrying the pGKl plasmids) were immune to each other (Table 2), implying that the plasmids encoded the resistance to the killer toxin. Certain strains of *K. lactis* (K43, WM37, K51, IFO 0648, and IFO 1090), however, were also resistant to the *K. lactis* killers in spite of the lack of pGKl plasmids. This may be explained by assuming the involvement of chromosomal gene(s) in the resistance. To prove this, a cross was made by mating a resistant strain K43 (**a** *trp*) with a sensitive strain L3α (α *met*), and tetrad analysis was conducted after sporulation. All of the 11 tetrads analyzed gave 2:2 segregation for mating types and auxotrophic (*met* and *trp*) markers (Table 5). Sensitivity and resistance to *K. lactis* killers (*K. lactis* IFO 1267 and tetrads 1A to 1D) also segregated in a ratio of 2:2, showing that the resistance of strain K43 to *K. lactis* killers was controlled by a single chromosomal gene, *kkr* (*Kluyveromyces* killer resistance). The hybrid between strains K43 and L3α was killed by *K. lactis* killers; therefore, the *kkr* gene was recessive. Linkage analysis indicated that the *kkr* gene was closely linked to the *trp* gene (PD: NPD:T = 5:0:6).

DISCUSSION

We searched for plasmids among 70 yeast strains of 17 different genera and found two novel DNA plasmids, pGKl1 (molecular weight, 5.4×10^6) and pGKl2 (molecular weight, 8.4×10^6), in *K. lactis* IFO 1267. These plasmids were characterized by a linear structure unlike the circular structure of bacterial plasmids and 2-μm DNA. The isolation of a linear plasmid-like DNA from a strain of *Streptomyces* sp. has been reported (13). Two linear DNA species associated with mitochondria have been demonstrated in a eucaryote, *Zea mays* (24). The present paper, however, is the first to disclose the existence of linear DNA plasmids in yeasts, although the intracellular localization remains obscure at present. The absence of the pGKl plasmids among the *K. lactis* strains tested, except *K. lactis* IFO 1267, indicates that the plasmids are not necessary for proliferation of cells, in analogy with the 2-μm DNA in *S. cerevisiae* (19).

K. lactis IFO 1267 was found to kill both sensitive and killer strains of *S. cerevisiae* at pH 5 to 7, but not at pH 4.5, in contrast to *S. cerevisiae* killers with dsRNA, which showed the killing at pH 4.5 to 5, but not at pH 7. In studying the killing action of various yeasts belonging to the genera *Saccharomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Debaryomyces*, *Candida*, and *Torulopsis*, Philliskirk and Young (23) have also demonstrated that a pH of 4.5 is optimum or close to optimum for the killing function of most yeasts. Thus, it appears that the killing at pH 5 to 7 is rather unique to the *K. lactis* killer. All of the 11 tested strains of *K. lactis* lacking the pGKl1 and pGKl2 plasmids were nonkillers. Tetrad analysis indicated an extrachromosomal inheritance of the killer character in association with the pGKl plasmids. In

TABLE 5. *Tetrad analysis of the cross K43 (a trp) × L3α (α met)*

Strain/tetrad	MT[a]	*met*	*trp*	Killing by:[b] *K. lactis* IFO 1267	Killing by:[b] *K. lactis* W600B	Killing by:[b] Tetrads 1A-1D
K. lactis K43	a	+	−	−	−	−
K. lactis L3α	α	−	+	+	−	+
Tetrad						
1A	α	+	−	−	−	−
1B	α	−	+	±	−	±
1C	a	−	−	+	−	+
1D	a	+	+	−	−	−
2A	a	−	+	+	−	+
2B	α	+	−	−	−	−
2C	α	+	−	−	−	−
2D	a	−	+	+	−	+
3A	a	−	−	−	−	−
3B	α	−	−	−	−	−
3C	a	+	+	+	−	+
3D	α	+	+	+	−	+
4A	α	+	−	+	−	+
4B	α	−	−	−	−	−
4C	a	−	+	+	−	+
4D	a	+	+	−	−	−
5A	α	+	−	−	−	−
5B	α	+	+	+	−	+
5C	a	−	+	+	−	+
5D	a	−	−	−	−	−
6A	—[c]	+	+	±	−	±
6B	α	−	+	+	−	+
6C	a	−	−	−	−	−
6D	α	+	−	−	−	−
7A	α	−	+	−	−	−
7B	α	+	−	+	−	+
7C	a	−	−	−	−	−
7D	a	+	+	+	−	+
8A	α	+	−	−	−	−
8B	a	−	+	+	−	+
8C	α	+	+	−	−	−
8D	a	−	−	+	−	+
9A	a	−	+	−	−	−
9B	a	−	−	+	−	+
9C	α	+	+	+	−	+
9D	α	+	−	−	−	−
10A	a	+	−	+	−	+
10B	α	−	+	+	−	+
10C	α	−	+	−	−	−
10D	a	+	−	−	−	−
11A	α	+	−	−	−	−
11B	a	−	−	−	−	−
11C	a	+	+	+	−	+
11D	α	−	+	+	−	±

[a] MT, Mating type.
[b] +, Killing; −, nonkilling; ±, weak killing.
[c] —, Nonmating.

addition, electrophoretic analysis has revealed that the killer character was not due to the presence of dsRNA, which encodes the production of protein toxin in *S. cerevisiae* (3). Thus, it seems highly probable that the killer character of *K. lactis* strains was under the control of the pGKl plasmids. To further evidence this, simultaneous curing of the killer character and the pGKl plasmids was attempted. The treatments with ethidium bromide and acriflavine were ineffective. Cycloheximide treatment (7) and growth at an elevated temperature (38°C) (34) have been reported to eliminate the dsRNA killer plasmid of *S. cerevisiae*, but they also gave negative results so far. Hereupon, it will be interesting to study whether nonkiller *K. lactis* or *S. cerevisiae* strains are transformed into killer strains with one or both of the pGKl1 and pGKl2 plasmids. Such transformation experiments are now in progress.

Young and Yagiu (36) have investigated various killer strains of different yeast genera and have found that killer yeasts other than *Saccharomyces* sp. do not carry dsRNA plasmids. Whether chromosomal gene(s) are responsible for the killings of such killer yeasts or whether the pGKl or other DNA plasmids are involved remains to be studied.

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ADDENDUM IN PROOF

Since this paper was submitted for publication, we have noticed that the pGKl plasmids and the killer phenotype were cured in the presence of ethidium bromide or by heat shock at 65°C. The details will be published elsewhere.

LITERATURE CITED

1. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275**:104–108.
2. **Bevan, E. A., and A. J. Herring.** 1976. The killer character in yeast: preliminary studies of virus-like particle replication, p. 153–162. *In* W. Bandlow, R. J. Schweyen, D. Y. Thomas, K. Wolf, and F. Kaudewitz (ed.), Genetics, biogenesis and bioenergenetics of mitochondria. Walter de Gruyter, New York.
3. **Bostian, K. A., J. Hopper, D. Rogers, and D. J. Tipper.** 1980. Translation analysis of the killer-associated virus-like particle dsRNA genome of *S. cerevisiae*: M dsRNA encodes toxin. Cell **19**:403–414.
4. **Clark-Walker, G. D.** 1972. Isolation of circular DNA

from a mitochondrial fraction from yeast. Proc. Natl. Acad. Sci. U.S.A. 69:388-392.

5. **Clark-Walker, G. D., and G. L. G. Miklos.** 1974. Localization and qualification of circular DNA in yeast. Eur. J. Biochem. 41:359-365.

6. **Cohen, J. D., T. R. Eccleshall, R. B. Needleman, H. Federoff, B. A. Buchffrer, and J. Marmur.** 1980. Functional expression in yeast of the *Escherichia coli* plasmid gene coding for chloramphenicol acetyltransferase. Proc. Natl. Acad. Sci. U.S.A. 77:1078-1082.

7. **Fink, G. R., and C. A. Styles.** 1972. Curing of a killer factor in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. 69:2846-2849.

8. **Fujii-Kawata, I., K. Miura, and M. Fuke.** 1970. Segments of genome of viruses containing double-stranded ribonucleic acid. J. Mol. Biol. 51:247-253.

9. **Gerbaud, C., P. Fournier, H. Blanc, M. Aigle, H. Heslot, and M. Guerineau.** 1979. High frequency of yeast transformation by plasmids carrying part or entire 2-μm yeast plasmid. Gene 5:233-253.

10. **Giudice, L. D., K. Wolf, P. Sassone-Corsi, and A. Mazza.** 1979. 2 μm covalently closed non-mitochondrial circular DNA in the petite negative yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. 172:165-169.

11. **Guerineau, M., C. Grandchamp, C. Paoletti, and P. Slonimski.** 1971. Characterization of a new class of circular DNA molecules in yeast. Biochem. Biophys. Res. Commun. 42:550-557.

12. **Gunge, N., and A. Tamaru.** 1978. Genetic analysis of products of protoplast fusion in *Saccharomyces cerevisiae*. Jpn. J. Genet. 53:41-49.

13. **Hayakawa, T., T. Tanaka, K. Sakaguchi, N. Otake, and H. Yonehara.** 1979. A linear plasmid-like DNA in *Streptomyces* sp. producing lankacidin group antibiotics. J. Gen. Appl. Microbiol. 25:255-260.

14. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.

15. **Hollenberg, C. P., P. Borst, and E. F. J. van Bruggen.** 1970. Mitochondrial DNA. V. A 25-μ closed circular duplex DNA molecules in wild-type yeast mitochondria. Structure and genetic complexity. Biochim. Biophys. Acta 209:1-15.

16. **Jazwinski, S. M., and G. M. Edelman.** 1979. Replication *in vitro* of the 2-μm DNA plasmid of yeast. Proc. Natl. Acad. Sci. U.S.A. 76:1223-1227.

17. **Johnston, J. R., and R. K. Mortimer.** 1959. Use of snail digestive juice in isolation of yeast spore tetrads. J. Bacteriol. 78:292.

18. **Livingston, D. M.** 1977. Inheritance of the 2 μm DNA plasmid from *Saccharomyces*. Genetics 86:73-84.

19. **Livingston, D. M., and D. M. Kupfer.** 1977. Control of *Saccharomyces cerevisiae* 2 μm DNA replication by cell division cycle genes that control nuclear DNA replication. J. Mol. Biol. 116:249-260.

20. **Luha, A. A., L. E. Sarcoe, and P. A. Whittaker.** 1971. Biosynthesis of yeast mitochondria. Drug effects on the petite negative yeast *Kluyveromyces lactis*. Biochem. Biophys. Res. Commun. 44:396-402.

21. **O'Conner, R. M., C. R. McArthur, and G. D. Clark-Walker.** 1975. Closed-circular DNA from mitochondrial enriched fractions of four petite negative yeasts. Eur. J. Biochem. 53:137-144.

22. **Ouchi, K., N. Kawase, S. Nakano, and H. Akiyama.** 1978. Stabilization of yeast killer factor by glycerol. Agric. Biol. Chem. 42:1-5.

23. **Philliskirk, G., and T. W. Young.** 1975. The occurrence of killer character in yeast of various genera. Antonie van Leeuwenhoek J. Microbiol. Serol. 41:147-151.

24. **Pring, D. R., C. S. Levings III, W. W. L. Hu, and D. H. Timothy.** 1977. Unique DNA associated with mitochondria in the "S"-type cytoplasm of male-sterile maize. Proc. Natl. Acad. Sci. U.S.A. 74:2904-2908.

25. **Royer, H., and C. P. Hollenberg.** 1977. *Saccharomyces cerevisiae* 2-μm DNA. An analysis of the monomer and its multimers by electron microscopy. Mol. Gen. Genet. 150:271-284.

26. **Shimotohno, K., and K. Miura.** 1973. Single-stranded RNA synthesis *in vitro* by the RNA polymerase associated with cytoplasmic polyhedrosis virus containing double-stranded RNA. J. Biochem. 74:117-125.

27. **Sinclair, J. H., B. J. Stevens, P. Sanghavi, and M. Rabinowitz.** 1967. Mitochondrial-satellite and circular DNA filaments in yeast. Science 156:1234-1237.

28. **Smith, D., P. Tauro, W. Schweizer, and H. O. Halvorson.** 1968. The replication of mitochondrial DNA during the cell cycle in *Saccharomyces lactis*. Proc. Natl. Acad. Sci. U.S.A. 60:936-942.

29. **Somers, J. M., and E. A. Bevan.** 1969. The inheritance of the killer character in yeast. Genet. Res. 13:71-83.

30. **Struhl, K., D. T. Stinchomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. U.S.A. 76:1035-1039.

31. **Sweeney, T. K., A. Tate, and G. R. Fink.** 1976. A study of the transmission and structure of double-stranded RNA associated with the killer phenomenon in *Saccharomyces cerevisiae*. Genetics 84:27-42.

32. **Taketo, M., S. M. Jazwinski, and G. M. Edelman.** 1980. Association of the 2-μm DNA plasmid with yeast folded chromosomes. Proc. Natl. Acad. Sci. U.S.A. 77:3144-3148.

33. **Tanaka, T., and B. Weisblum.** 1975. Construction of a colicin E1-R factor composite plasmid in vitro: means for amplification of deoxyribonucleic acid. J. Bacteriol. 121:354-362.

34. **Wickner, R. B.** 1974. "Killer character" of *Saccharomyces cerevisiae*: curing by growth at elevated temperatures. J. Bacteriol. 117:1356-1357.

35. **Wickner, R. B., and M. J. Leibowitz.** 1976. Chromosomal genes essential for replication of a double-stranded RNA plasmids of *Saccharomyces cerevisiae*: the killer character of yeast. J. Mol. Biol. 105:427-433.

36. **Young, T. W., and M. Yagiu.** 1978. A comparison of the killer character in different yeasts and its classification. Antonie van Leeuwenhoek J. Microbiol. Serol. 44:59-77.