Efficient isolation of the linear DNA killer plasmid of *Kluyveromyces lactis*: evidence for location and expression in the cytoplasm and characterization of their terminally bound proteins

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ABSTRACT

Differential centrifugation of an osmotic lysate of *K. lactis* protoplasts showed that the linear DNA killer plasmids of *K. lactis*, pGKL1 and pGKL2, are almost exclusively present in the cytoplasmic fraction. This fractionation procedure allows the rapid isolation of large amounts of plasmid DNA without contamination by chromosomal and mitochondrial DNA. With these DNA preparations the size of the terminally bound proteins was estimated to be 28 and 36 kDal for pGKL1 and pGKL2, respectively. The entire pGKL1 sequence (except for 21 base pairs at the right terminus) was cloned in a shuttle vector that permits autonomous replication in the nucleus of *K. lactis*. However, killer gene expression could not be established in transformants. In connection with the observed cytoplasmic localization, this result suggests that gene expression of the killer DNA plasmids is entirely cytoplasmic.

INTRODUCTION

Some strains of the yeast *K. lactis* secrete a protein toxin that kills sensitive strains of the same and some other yeast species (1). Killer protein producing strains are resistant to the toxin. The killer and immunity proteins are encoded by two linear dsDNA plasmids, pGKL1 and pGKL2 (1, 2). Transfer of these plasmids from *K. lactis* into other yeast species demonstrated that pGKL1 and pGKL2 can also replicate stably and express their killer phenotype in other hosts (3, 4, 5). Deletion analysis and plasmid curing has shown that pGKL1 codes for killer and immunity protein subunits, whereas pGKL2 is probably required for maintenance of pGKL1 (2, 6). More complex interrelationships between pGKL1 and pGKL2 may be expected but their linearity and the lack of an efficient transformation procedure hampers a rapid functional analysis of their gene structure.

The termini of pGKL1 and pGKL2 carry unique inverted terminal repeats of 202 and 184 bp, respectively (7, 8). Linear DNA elements with such a terminal DNA sequence often have proteins covalently attached at their 5' ends. Examples are maize mitochondrial plasmids (9), *Steptomyces rochei* plasmids (10), Pneumococcal bacteriophage Cp-1 (11), *Bacillus subtilis* phage ss29 (12,
13) and Adenovirus (14, 15). The proteins bound at the termini of Adenovirus and $\Phi$ 29 DNA are involved in protein priming at initiation of a displacement mode of DNA replication (14, 16, 17). Indirect evidence has been obtained that the termini of pGKL1 and pGKL2 do have a similar structure (7, 18) and it was suggested that their terminal proteins might be very small (18). This paper describes the rapid isolation of large amounts of pure pGKL1 plus pGKL2 from the cytoplasmic fraction of osmotically lysed K. lactis protoplasts. Subsequent separation of pGKL1 and pGKL2 by SDS-agarose gelelectrophoresis enabled us to label the terminal proteins and determine the molecular weights of the proteins bound to each of these killer plasmids.

From meiotlic segregation data (1) and fluorescence DNA staining of rho" mutant cells of a Saccharomyces cerevisiae strain that harbours pGKL1 and pGKL2 (4) it was inferred that a significant fraction of the killer DNA plasmids is present in the cytoplasm. These observations are supported by the fractionation study presented in this paper. Our data suggest that probably all plasmid DNA is located in the cytoplasm.

In a first attempt to study gene expression of killer DNA plasmids in more detail, we have hooked almost complete pGKL1 DNA to an E. coli - K. lactis shuttle vector that replicates in the yeast nucleus. Expression of killer activity of these cloned pGKL1 sequences was tested in a killer" K. lactis strain that harbours pGKL2. No activity could be observed. As will be discussed, these results suggest that the genes encoded by these linear DNA plasmids are uniquely expressed in the cytoplasm.

MATERIALS AND METHODS

Yeast strains and plasmids

K. lactis strain SD11 lac4 trpl was obtained from C.P. Hollenberg (19). SD11 appeared to contain pGKL1 and pGKL2 and we have used this strain for isolation of killer plasmid DNA.

Amongst thousand tested SD11 colonies we found eight spontaneous killer" mutants. Four had lost pGKL1 only (SD11 kl") and four had lost both pGKL1 and pGKL2 (SD11 kl"k2"). A SD11 kl"k2" strain harbouring plasmid pGL2 (TRP1 KARS2 G418R; 20) was used as indicator strain for testing killer activity. The TRP1 and KARS2 containing sequences of pEM384 (Fig. 7) are derived from pEK27 (20). The sequence of pEM384 that confers G418 resistance to K. lactis was originally derived from pKT071 (21). The pGKL1 insert in pEM384 was obtained by joining two ScaI fragments after cloning a partial ScaI digest into the Smal site of pUC18. Restriction enzyme analysis and DNA
sequencing confirmed that the pGKL1 insert in pEM384 contains all Scal fragments in the correct orientation as present in native pGKL1 DNA. It turned out that at the left terminus pGKL1 was cloned starting at base pair 1 (7, 8) while at the right end the terminal Scal fragment of 21 bp was not included.

Media

The complex medium for growth of yeast was YEPD (1% yeast extract, 2% peptone, 2% glucose). The minimal medium for growth of yeast transformant colonies contained 0.67% yeast nitrogen base, 2% glucose and all amino acids (except for tryptophane) at 100 µg per ml each. Killer activity was tested on gal-agar (1% yeast extract, 1% peptone, 2% galactose, 0.05 M KH2PO4 pH4.8; 2) supplemented with 200 µg per ml G418 (19).

Transformation of K. lactis

K. lactis strains were transformed by using the lithium acetate method of Ito et al. (22).

SDS-agarose gelelectrophoresis

Agarose gelelectrophoresis of killer DNA protein complexes was performed in 0.9% agarose in the presence of SDS. Both sample buffer, agarose gel and TBE running buffer contained 0.2% SDS. After electrophoresis the gel was soaked in water to remove SDS and nucleic acids were stained with EtBr (0.5 µg/ml).

Isolation of killer DNA-protein complexes

The standard isolation procedure for pGKL plasmids is described by Gunge et al. (1).

The isolation of pGKL plasmids according to our osmotic lysis procedure is as follows. K. lactis cells were grown in 200 ml YEPD to a density of 2 x 10^8 cells per ml. Cells were converted to protoplasts as described by Das and Hollenberg (19). Protoplasts were washed once in 1.2 M sorbitol. The protoplast pellet was suspended in 13 ml ice-cold TM buffer (10 mM Tris-HCL pH 8.0, 10 mM MgCl2) which contained 1 mM phenyl methyl sulfonyl fluoride (PMSF) and left on ice for 5 min. This results in osmotic lysis of 85-95% of the protoplasts. For preparative isolation of killer plasmids this lysate was centrifuged directly for 15 min at 15,000 rpm in a Sorvall SS34 rotor to remove intact cells, cellular debris, nuclei, mitochondria and vacuoles. The supernatant was layered on a 1 ml CaCl cushion (40% (w/v) in TM buffer) and run in a MSE SW 6 x 14 ml Ti rotor for 90 min at 40,000 rpm at 10°C. The CaCl cushion that contained the killer plasmids was adjusted to 0.2% SDS and applied to a preparative SDS-agarose gel. The plasmids were isolated from the gel by electro elution and after adding 0.2% SDS they were precipitated with
ethanol (2.5 vol.) and 0.3M Sodium acetate. The DNA precipitate was dissolved in 60 \mu l TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

Iodination of DNA-protein complexes with \(^{125}\text{I}\)

20 \mu l of a purified plasmid preparation was adjusted to 0.1\% sarkosyl and labeling was carried out with 100 \mu Ci \(^{125}\text{I}\) in Iodogen-coated tubes as described by Schouten (23). Next, DNA-protein complexes were ethanol precipitated, dissolved in TE containing 0.2\% SDS and subjected to Biogel A-50 m (Biorad) chromatography in the presence of 0.2\% SDS. To remove any labeled unbound material, the plasmid DNA-containing fractions were pooled, ethanol precipitated and further purified by SDS-agarose gelelectrophoresis. Finally, DNA-protein complexes were dissolved in 20 \mu l 20 mM Tris-HCl pH 7.6.

S1 nuclease

Treatment with S1 nuclease was performed as described by Schouten (23). After denaturing DNA for 5 min at 95°C 180 \mu l S1 buffer was added and the sample was incubated for 30 min at 65°C with 330 units S1 nuclease. To the samples 4 \mu l 5 M NaCl and 4 \mu l cytochrome C was added and after the addition of 800 \mu l acetone proteins were precipitated overnight at -20°C.

Proteinase K

Proteinase K treatment was performed in 0.2\% SDS with 0.5 \mu g proteinase K per ml at 37°C for 45 min.

Protein gelelectrophoresis

Protein gelelectrophoresis was performed in 12\% SDS polyacrylamide gels as described by Laemmli (24).

RESULTS

Detection of pGKL plasmids on SDS-agarose gels

In our hands pGKL plasmids, isolated according to the standard procedure described by Gunge et al. (1), cannot be detected after agarose gelelectrophoresis unless the samples were treated with proteases (Fig. 1A). This observation agrees with the gel migration behaviour of other DNA elements with covalently attached proteins (9,11-14). The DNA is prevented from entering the agarose gel, presumably by aggregation that occurs by protein-protein interaction of the attached proteins (11, 12, 14). Solubilisation of the proteins by adding SDS to the sample buffer does not result in proper migration of the DNA-protein complexes (9, 11-14). However, we noticed that the addition of 0.2\% SDS to agarose gel and running buffer too enables gelelectrophoretic analysis of pGKL plasmids (Fig. 1B). Therefore, SDS-agarose gelelectrophoresis has been used throughout this work for both analytical and preparative purposes.
Isolation and purification of pGKL plasmids from the cytoplasm of osmotically lysed K. lactis protoplasts

After total lysis of K. lactis protoplasts with SDS the standard isolation procedure of pGKL plasmids involves a high salt precipitation step to remove cellular debris, protein and high molecular weight DNA (1). However, as shown in Fig. 1C, most killer plasmid DNA is lost at this stage in the precipitate.

Taking advantage of the observation that a significant fraction of killer plasmids is located in the yeast cytoplasm (4), we have devised a novel purification procedure (Table I, Fig. 2). K. lactis cells are converted to protoplasts and subsequently osmotically lysed in buffer, containing 10 mM Mg\textsuperscript{2+}. This leaves nuclei and mitochondria largely intact. Together with unlysed cells and cellular debris they are removed by low speed centrifugation. About 95% of killer plasmid DNA is recovered in the cytoplasmic fraction (Fig. 2, slots 2, 3). Higher speed centrifugation removes almost all remaining chromosomal contamination (Fig. 2, slots 4, 5). Subsequently, pGKL plasmids are centrifuged into a CsCl cushion, thereby concentrating them and separating them from cytoplasmic proteins. After adding 0.2% SDS to the CsCl fraction pGKL1 and pGKL2 are separated on a preparative SDS-agarose gel. Since proteinase K treatment is required to enter an agarose gel that does not contain SDS (Fig. 3), we conclude that this purification procedure for pGKL1 and
Table I. Purification of killer DNA by differential centrifugation of osmotically lysed protoplasts

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Sample</th>
<th>Yield of killer DNA</th>
<th>Purification</th>
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<tbody>
<tr>
<td>Old K. lactis culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 ml protoplasts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmotic shock</td>
<td></td>
<td></td>
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<tr>
<td>In 13 ml TM</td>
<td>1</td>
<td>15 µl</td>
<td>100%</td>
</tr>
<tr>
<td>5 min 5,000 rpm</td>
<td>P(Ellet) 5,000</td>
<td>2 15 µl&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~95% ~50%</td>
</tr>
<tr>
<td></td>
<td>S(Supernatant) 5,000</td>
<td>3 15 µl&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~93% ~95%</td>
</tr>
<tr>
<td>15 min 15,000 rpm</td>
<td>P&lt;sub&gt;15,000&lt;/sub&gt;</td>
<td>4 15 µl&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S&lt;sub&gt;15,000&lt;/sub&gt;</td>
<td>5 15 µl&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~93% ~95%</td>
</tr>
<tr>
<td>90 min 40,000 rpm on 1 ml CsCl (&lt;.ct=1.3&gt;)</td>
<td>S&lt;sub&gt;40,000&lt;/sub&gt;</td>
<td>6 15 µl&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;5%</td>
</tr>
<tr>
<td></td>
<td>CsCl</td>
<td>8, 9, 10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>~80% ~99%</td>
</tr>
<tr>
<td></td>
<td>Ribosome</td>
<td>7 15 µl&lt;sup&gt;e&lt;/sup&gt;</td>
<td>~10%</td>
</tr>
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<sup>a</sup> Samples were adjusted to 0.2% SDS and subjected to agarose gelelectrophoresis in the presence of 0.2% SDS (see Fig. 2).<sup>b</sup> The purification of killer DNA was determined by agarose gelelectrophoresis and is expressed as the ratio of killer DNA to chromosomal and mitochondrial DNA.<sup>c</sup> Pellets were suspended in 13 ml TM buffer containing 0.2% SDS.<sup>d</sup> The CsCl fraction had a volume of 900 µl, samples 8, 9 and 10 were 1, 2 and 5 µl, respectively.<sup>e</sup> The ribosome pellet was suspended in 3 ml TM buffer containing 0.2% SDS and 15 µl of this fraction was analysed.
pGKL2 leaves their terminally-bound proteins largely intact.

Direct evidence for attachment of proteins to the termini of pGKL1 and pGKL2
SDS-agarose gel purified plasmid DNA-protein complexes were cut with restriction enzymes to proof that proteins are attached to their termini. pGKL1 was digested with PstI, producing a fragment of 6.44 kb and terminal fragments of 1.55 and 0.88 kb (8, 25). pGKL2 was digested with EcoRI, producing fragments of 3.7, 3.4, 1.4, 1.25 and 1.1 kb and terminal fragments of 0.86 and 0.8 kb (1, 7). When these digestion mixtures were run on an agarose gel in the absence of SDS, only the terminal fragments did not enter the gel (data not shown). More direct evidence for attachment of protein to the termini of pGKL1 and pGKL2 was obtained by labeling proteins with $^{125}$I and analysis of labeled restriction enzyme digests on SDS-agarose gels. SDS-agarose gel purified DNA-protein complexes were labeled with $^{125}$I, using the Iodogen method. After removal of labeled unbound material by Biogel A-50 m chromatography in the presence of 0.2% SDS, followed by SDS-agarose gel
Bacteriophage λ DNA, digested with HindIII and uniformly labeled with $^{32}$P by nick translation (sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 and 0.125 kb), slots 1, 7. Bacteriophage fd 109 DNA, digested with HaeIII and uniformly labeled with $^{32}$P (sizes 2.5, 1.6, 0.85, 0.59, 0.46, and 0.38 kb), slots 2, 8. PstI digested $^{125}$I labeled pGKL1 DNA-protein complex uniformly labeled with $^{32}$P by nick translation, slot 3. PstI digested $^{125}$I labeled pGKL1 DNA-protein complex, slot 4. EcoRl digested $^{125}$I labeled pGKL2 DNA-protein complex, slot 5. EcoRl digested $^{125}$I labeled pGKL2 DNA-protein complex uniformly labeled with $^{32}$P, slot 6. A and B indicate the position of the terminal pGKL1 PstI fragments and C indicates the position of the terminal EcoRl fragments of pGKL2. The amount of DNA applied to slots 3 and 6 was approximately 1% of the DNA applied to slots 4 and 5.

electrophoresis, $^{125}$I-labeled pGKL1 and pGKL2 DNA-protein complexes were digested with PstI and EcoRl, respectively. Thereafter, DNA of part of both restriction enzyme digests was uniformly labeled with $^{32}$P by nick translation. Samples of PstI and EcoRl digested DNA-protein complexes, labeled with $^{125}$I only or labeled with $^{125}$I and $^{32}$P were analysed by SDS-agarose gel electrophoresis (Fig. 4). As expected, only bands that migrate at a position corresponding to the terminal 1.55 and 0.96 kb PstI fragments of pGKL1 are $^{125}$I labeled (Fig. 4, slot 4). Similarly, $^{125}$I label can only be detected at the position of the terminal 0.86 and 0.8 kb EcoRl fragments of pGKL2, which are not well separated on this gel (Fig. 4, slot 5). As is most evident for pGKL1 fragment B, all $^{32}$P labeled terminal fragments seem to resolve in two bands. The upper bands run at the same velocity as the $^{125}$I labeled fragments (compare lanes 3 and 4 at position B and lanes 5 and 6 at position C in Fig. 4). Since the amount of $^{125}$I, $^{32}$P double labeled DNA present in lanes 3 and 6
is less than 1% of the $^{125}$I labeled DNA in lanes 4 and 5, we infer that the upper bands also represent $^{32}$P labeled material. Moreover, treatment of the nick translated samples with proteinase K resulted in the disappearance of the upper bands (results not shown). From these data we conclude that the upper bands represent restriction fragments still containing terminal protein, whereas the lower bands have lost the protein and therefore migrate slightly faster. Apparently some proteolysis has occurred at a stage after the purification described in the previous section.

**Determination of the molecular weight of the terminal proteins**

Isolated pGKL DNA-protein complexes were iodinated and purified as described in the previous section. Samples, after treatment with S1 nuclease, were subjected to SDS-polyacrylamide gelelectrophoresis. The results for pGKL1 and pGKL2 DNA-protein complexes are shown in Fig. 5 and 6, respectively. Without prior degradation of the DNA by S1 nuclease all radioactivity stayed on top
Fig. 6. Analysis of the pGKL2 DNA-protein complex by SDS-polyacrylamide gel electrophoresis. 125I labeled protein marker mixture, the sizes are indicated in kilo dalton, slots 1, 2 and 7. 125I labeled pGKL2 DNA-protein complex treated with 1 and 5 µg proteinase respectively, slots 3 and 4. Untreated DNA-protein complex, slot 5. DNA-protein complex treated with SI nuclease, slot 6.

of the gel (Fig. 5, slot 2; Fig. 6, slot 5). On the other hand proteinase K treatment resulted in degradation of protein to very fast migrating material (Fig. 5, slot 1; Fig. 6, slots 3 and 4). After treatment of both DNA-protein complexes with SI nuclease under conditions that keep DNA denatured, each complex appeared to carry one unique protein. Their molecular weights differed and were about 28 and 36 kDa for pGKL1 and pGKL2, respectively (Fig. 5, slot 3; Fig. 6, slot 6). It remains to be established whether there is any relationship between these proteins.

Expression of cloned pGKL1 DNA in K. lactis

Since we have observed that most, and probably all killer DNA plasmids, are located in the cytoplasm of yeast cells, the possibility must be considered that they code for proteins required for transcribing and replicating their DNA. However, very little is known about the structure and expression of genes present on pGKL1 and pGKL2 since mutant plasmids are not easily obtained (2, 6). Therefore, we carried out a pilot experiment to test whether gene expression of pGKL plasmids can be studied in an alternative way. For this purpose plasmid pEM384 was constructed, which carries the following DNA sequences (Fig. 7). (1) The complete pGKL1 DNA sequence except for a 21 bp
Fig. 7. Physical structure of plasmid pEM384. The different sequences contained by pEM 384 are described in the text. H, HindIII; P, PstI; E, EcoRl; B, BamHl; X, Xhol.

Scal fragment at the right end terminus of pGKL1 (8, 25). The cloned sequence contains all four open reading frames of pGKL1 (8). (i) A sequence allowing autonomous replication of pEM384 in the yeast nucleus (KARS 2; 19). (ii) The S. cerevisiae TRP1 gene to introduce the plasmid into K. lactis strain SD11 lac' trpl by transformation (19). (iv) A DNA fragment derived from Tn903 that confers resistance to kanamycin in E. coli and to G418 in K. lactis (19, 26). (v) The pUC18 vector for construction and propagation of the plasmid in E. coli.

We have tested whether killer activity is phenotypically expressed by pEM384 in killer− strain SD11 kl−. SD11 kl− is a derivative of SD11 lac' trpl that has spontaneously lost pGKL1 and still harbours pGKL2. Killer activity was assayed by streaking transformant colonies on gal plates (2) containing G418 to keep selective conditions for the recombinant plasmid and using K. lactis SD11 kl−k2− that harbours plasmid pGKL2 (G418R, 20), as indicator strain. SD11 kl+k2+ containing plasmid pEM384 exhibited strong killer activity with these assay conditions. However, it appeared that pEM384 did not restore detectable killer activity to SD11 kl−. Probably this observation is due to a lack of transcription of pGKL1 genes in the yeast nucleus.

DISCUSSION

Terminal proteins of pGKL plasmids

Previously indirect evidence was presented that proteins are attached to the termini of pGKL plasmids. (i) The 5' ends of these linear plasmids are not accessible for labeling with 32P by polynucleotide kinase (7). (ii) Terminal
restriction fragments showed altered electrophoretic mobilities after treatment with proteases (18). In the present study we have demonstrated directly, by labeling purified DNA-protein complexes with $^{125}$I, that proteins are bound to terminal restriction fragments. It was suggested by Kikuchi et al. (18) that these proteins are very small. However, we have estimated the molecular weights of these proteins at about 28 and 36 kDal for pGKL1 and pGKL2, respectively. These contradictory results are probably explained by the different methods used to isolate pGKL plasmids. According to the standard procedure, yeast protoplasts are lysed with SDS and subsequently proteins and high molecular weight DNA are precipitated by adding a high concentration of salt (1, 18). Fig. 1C shows that most pGKL plasmids are also precipitated, probably due to their terminally attached proteins. Thus, this method may preferentially select for plasmids which have lost most or all of their proteins by proteolysis. As we experienced with proteinase K, proteolysis is not completely inhibited by SDS and NaCl in SDS lysates (data not shown). Therefore, proteolysis might have occurred since SDS lysis of protoplasts also destroys yeast vacuoles that contain many proteases (27). During isolation of pGKL plasmids according to the method described in this paper, osmotic lysis of protoplasts followed by differential centrifugation, the integrity of these organelles is better maintained. Thus, DNA-protein complexes are probably less exposed to proteolytic attack by these cellular proteases when isolated according to this procedure.

In all likelihood the terminal proteins of pGKL plasmids fulfill a function in protein priming of DNA replication (28), similar to the proteins covalently attached to the termini of adenovirus and ϕ29 DNA (16, 17, 29). In the case of adenovirus and ϕ29 a free terminal protein (or precursor molecule) binds, after associating with a DNA polymerase, to a nucleotide that becomes the 5' end of the DNA sequence. The generation of this primer for DNA synthesis is dependent on sequences within the inverted terminal repeats (16, 29). An identical mechanism is implicated for priming of replication at both ends of these and similar linear DNA elements. This probably also accounts for the S1 and S2 plasmids which are together present in mitochondria of S-type male sterile maize cytoplasm (9). S1 and S2 carry identical inverted terminal repeats (30), suggesting that the same factors are involved in protein priming of both S1 and S2. In contrast to S1 and S2 plasmids, the sequence of the inverted terminal repeats of pGKL1 and pGKL2 DNA is entirely different. Therefore, it is not surprising when different factors are involved in their replication, in agreement with our observation that both plasmids carry dif-
different, although perhaps structurally related, terminal proteins. However, since the number of plasmid copies per cell is about the same for pGKl1 and pGKL2, it is not immediately obvious why different factors are required for initiation of DNA replication of both plasmids.

Cytoplasmic localization of pGKL plasmids

Several lines of evidence have pointed to a cytoplasmic localization of at least part of the killer DNA plasmids. (i) After the introduction of killer plasmids into a S. cerevisiae rho° strain, fluorescence appears in the cytoplasm of cells after staining DNA with a fluorescent dye (4). (ii) The data of cytoduction experiments indicated a cytoplasmic inheritance of pGKL plasmids (3).

The data presented in this paper suggest that pGKL plasmids are exclusively located and expressed in the yeast cytoplasm. Firstly, osmotic lysis of K. lactis protoplasts followed by differential centrifugation of nuclei and other cellular organelles showed that at least 95% of killer DNA plasmids is found in the cytoplasmic fraction. We think that the small amount of plasmid DNA that cosediments with the nuclei must be attributed to the imperfection of differential centrifugation. Secondly, like mitochondrial DNA, their high AT content may result from a non-nuclear replication as pointed out by Stark et al. (25). Finally, we have tested whether killer toxin activity is phenotypically expressed by pGKL1 cloned into a plasmid that replicates autonomously in K. lactis. The structure of the terminal sequences required for replication of linear pGKL1 DNA is destroyed in this circular chimeric plasmid (pEM384). Therefore, only KARS2 and similar sequences on pGKL1 (31), which act as replication origins in the nucleus, allow replication of pEM384 in K. lactis. After introducing the plasmid into a K. lactis strain that also harboured pGKL2, no expression of killer activity was observed although the TRPl and G418F genes were clearly expressed. Possibly killer toxin is expressed at a level that cannot be detected due to the lower number of pEM384 plasmid copies in comparison to native pGKL1. However, all evidence taken together, we favour the possibility that genes coding for killer protein are not expressed by pEM384 at all, due to an absolute dependency on cytoplasmic factors. pGKL2 seems to be required for maintenance of pGKL1 in K. lactis (2, 6). Therefore, we suggest that pGKL2 codes for a specific transcription factor of pGKL1 and pGKL2 genes.

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