The DNA and RNA polymerase genes of yeast plasmid pGKL2 are essential loci for plasmid integrity and maintenance

Raffael Schaffrath, Surinder M. Soond and Peter A. Meacock

Novel recombinant plasmids derived from the *Kluyveromyces lactis* killer plasmid k2 have been constructed to study plasmid biology and gene function. *In vivo* recombination between native resident k2 and suitable disruption vectors, employing the KITRP1 gene fused to a plasmid promoter as selection marker, yielded ORF2 and ORF6 deletion plasmids at high frequencies. As judged from Southern hybridization and plasmid restriction mapping analyses, these novel hybrids, termed rk2/2 and rk2/6, respectively, carry deletions in their putative DNA (ORF2) and RNA (ORF6) polymerase structural genes with central regions replaced by the input marker DNA. Long-term selection for *TRP1* over 350 generations of growth did not favour maintenance of hybrids over wild-type k2. Thus, neither rk2/2 nor rk2/6 was fully functional and able to displace parental k2, indicating that both target genes are essential for plasmid integrity or maintenance. Recombinant plasmids were reduced in copy number relative to k2 with rk2/2 more drastically affected than rk2/6 implying a direct involvement of the ORF2 product in plasmid replication and an indirect maintenance function for the ORF6 gene product.

**Keywords:** *Kluyveromyces lactis*, killer system, linear plasmids, DNA/RNA polymerases, gene disruptions

INTRODUCTION

Linear DNA plasmids have been identified for various yeast genera such as *Debaryomyces*, *Pichia*, *Wingea*, *Kluyveromyces* and *Saccharomyces* and shown to share many common features. They are extremely A + T-rich (Stark et al., 1990; Hishinuma & Hirai, 1991), partially cross-hybridize to each other (Cong et al., 1994; Bolen et al., 1994) and show structural and phylogenetic relatedness to adenovirus in carrying terminal inverted repeats (TIRs) with covalently attached terminal proteins (TPs) (Meinhardt et al., 1990; Meinhardt & Rohe, 1993; Stark et al., 1990; Rohe et al., 1992; Guge et al., 1993). In terms of gene function, the best-characterized of these yeast episomal systems is the cytoplasmically localized killer plasmid pair, k1 and k2, of the dairy yeast *Kluyveromyces lactis* (for review see Stark et al., 1990). The smaller plasmid k1 (8-9 kb) carries four ORFs (Hishinuma et al., 1984) with gene functions involved in plasmid replication and expression of killer and immunity phenotypes (Stark & Boyd, 1986; Tokunaga et al., 1987). Genetic analysis has shown that plasmid k2 (13-5 kb), which carries ten ORFs, provides essential maintenance functions presumably involved in transcription and replication of both plasmids (Stark et al., 1990; Schaffrath & Meacock, 1995; Schaffrath et al., 1995). Thus, k2 appears to encode an initiation factor for k1/k2 replication as well as putative helicase and DNA/RNA polymerases (McNeel & Tamanoi, 1991; Tommasino, 1991; Tommasino et al., 1988; Wilson & Meacock, 1988; Stark et al., 1990). Upstream conserved sequence (UCS) motifs preceding all plasmid genes have been shown to possess promoter activity and have been exploited for gene disruption analysis. In fact k1/k2-targeted integration of foreign DNA was only possible when markers, i.e. *Saccharomyces cerevisiae* LEU2 and *Escherichia coli* Tn503-derived G418<sup>R</sup>, were transplanted as UCS-fusion genes. Like native k1 and k2, the resulting hybrids exhibited identical characteristics in having a linear structure with TPs attached, cytoplasmic location and high stability (Kämper et al., 1991; Tanguy-Rougeau et al., 1990; Schaffrath et al., 1992). This allelic replacement procedure *in vivo* has proven useful for the study of both basic and applied aspects of linear plasmid
biology. In this manner, k1ORF2 and k2ORF1 have been identified as dispensable genes (Kämper et al., 1991; Schaffrath et al., 1992) and potential loci for heterologous gene expression (Meinhardt et al., 1994). Here, we report a gene-disruption analysis of two k2 genes, the putative DNA and RNA polymerase genes ORF2 and ORF6, respectively, using a UCS–TRPl fusion marker and show both loci to be essential for plasmid integrity and/or maintenance.

**METHODS**

**Strains, plasmids and general methods.** All strains and plasmids used and generated in the course of this study are listed in Tables 1 and 2. Basic yeast methods and growth media,YPD and SD, were as described by Sherman (1991). Yeast transformations were carried out according to Gietz et al. (1992) using 5 μg transfer vectors pAR5 and pMS202/600 completely digested as indicated to release recombination cartridges (Fig. 1). Routine bacterial growth conditions and recombinant DNA methodology were adopted from Sambrook et al. (1989). DNA sequence was determined by the dideoxy method (Sanger et al., 1977) using the T7 Sequencing kit (Pharmacia). Standard PCRs were carried using synthetic oligonucleotide primers (see below), all four dNTPs at 0·2 mM each and Vent DNA polymerase (New England Biolabs) according to the supplier’s instructions. Synthetic oligonucleotides were: k2 pos. 10723–10740 (Tommasino et al., 1988), FW6 5’ GGATCCAGAAA-TAGGTAAGTAC 3’ (k2ORF6); and k2 pos. 11280–11299, FW7 5’ CATATGAATGAAATATTATTTC 3’ (k2ORF7).

**Construction of k1- and k2-targeted disruption vectors**

**pAR5.** Prior to the construction of k2-gene targeting vectors, plasmid pAR5 was generated for specific disruption of k1ORF2 in control experiments. In pAR5 the 1·6 kb ScLEU2 gene fragment of pAR4 (J. Schründner & F. Meinhardt, personal communication) was replaced by the 0·7 kb NsiI/BamHI KlTRPl gene, fragment of pSS9 (S.M. Soond & P.A. Meacock, unpublished). For convenience, the external pUC-based HindIII site was converted into an NdeI site by restriction, Klown fill-in and re-ligation (Fig. 1a). Thus, digestion of pAR5 with NdeI and EcoRI releases the k1ORF2 disruption cassette for yeast transformation experiments.

**pMS600.** Plasmid pMS600, a targeting vector for disruption of k2ORF6, was constructed as follows. A 0·8 kb NsiI k2 fragment, obtained from pRS23, a k2 subclone derived from pGKF202, was cloned into the single NsiI site of pUCBM21. In the resultant pRS27, this k2 segment (pos. 7317–8060, Tommasino et al., 1988) carries ORF5 and a short 5’ ORF6 region representing the 26 amino terminal residues of the putative RNA polymerase. This ORF6 segment was flanked downstream by the 1·9 kb PstI/BamHI k1UCS2–LEU2 fusion gene from pRS1 to give pRS36. The use of template pGKF202 and primers FW6/7 (see above) in PCR yielded a second 0·6 kb k2 fragment which was subcloned into Smal-cut pUC13 (pRS16). This k2 fragment carries the complete ORF7 and the 3’ terminal 0·2 kb of ORF6 (k2 pos. 10723–10906, Tommasino et al., 1988). Subcloning this fragment into BamHI-restricted pRS36 generated the vector pMS60. Finally, pMS600 was constructed by replacement of the ScLEU2 marker with the KITRP1 gene from pAR5. Thus, in pMS600 the k1UCS2 element is fused in-phase to TRPI. Allelic replacement of k2ORF6 will occur when yeast cells are transformed with pMS600 digested with HindIII and NdeI (Fig. 1b).

**pMS202.** Plasmid pMS202, a targeting vector for disruption of k2ORF2, was constructed as follows. Replacement of the k2 sequence of pRS66, excised with HindIII and PstI, with a terminal 1·0 kb HindIII/NsiI fragment of pICk2S (Table 2) resulted in pRF1. The k2 segment of pRF1 carries k2ORF1 preceded by a partial TIR sequence and the 3’ terminal 0·2 kb of k2ORF2 (k2 pos. 117–1106). pRF2 was generated by replacing the ScLEU2 marker with the KITRP1 gene from pAR5 as outlined above. Part of the ampicillin resistance gene of pRF2 was released upon Smal/ScaI double restriction and functionally reconstituted with the 3·5 kb ScaI fragment of pICk2S to give pMS200. This second k2 segment contains almost all of ORF3 (k2 pos. 3889–5655) and the 5’ terminal 0·7 kb region of ORF2 (k2 pos. 3221–5655, Tommasino et al., 1988). Thus, the disruption cartridge of pMS202 released by HindIII digestion was expected to promote in vivo recombination and allelic replacement of ORF2 by the TRPI marker gene (Fig. 1c).

<table>
<thead>
<tr>
<th>Table 1. Strains</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
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<tr>
<td><strong>K. lactis</strong></td>
</tr>
<tr>
<td>AW1137</td>
</tr>
<tr>
<td>SD801</td>
</tr>
<tr>
<td>KRS13</td>
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<tr>
<td>MS20</td>
</tr>
<tr>
<td>MS60</td>
</tr>
</tbody>
</table>
Yeast plasmid pGKL2 DNA and RNA polymerase genes

As pAR4, but 1.6 kb ScLEU2 replaced by 0.7 kb KlTRPI

Linear kl ORF2' plasmid (AORF2 Δ:Δ Kl TRP I)

pUCBM21 with k2 insert of pGKF202 (pos. 7314-11008)

pUCBM21 with 0.8 kb k20RFhpanning fragment from pRS23

pUC13 with 0.6 kb k20RF7 PCR fragment

pRS27 with kl UCS2LEUZ fusion marker from pRSl

pRS36 with second k20RF7-spanning fragment of pRSl6

pMS6O with ScLEU2 replaced by KlTRPl from pAR5

Linear k20RF6' plasmid (AORF6 Δ:Δ Kl UCS2KlTRP I)

pRS36 with k20RF5 replaced by k20RF1 from pICk2S

pRF2 with ScLEU2 replaced by KI TRP I from pAR5

pMS202 with second k20RF2/3-spanning fragment from pICk2S

rk2/2 Linear k20RF2a plasmid (ΔORF2 Δ:Δ Kl UCS2KI TRP I)

- **Table 2. Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pUC13/BM21</td>
<td>General cloning vectors</td>
<td>Pharmacia/Boehringer</td>
</tr>
<tr>
<td>k1/k2</td>
<td>Linear killer plasmids (8.9/13.5 kb) from <em>K. lactis</em></td>
<td>Gunge <em>et al.</em> (1981)</td>
</tr>
<tr>
<td>pKLK109</td>
<td>pUC with internal k1 fragment</td>
<td>Stark <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>pACT3</td>
<td>pUC with KI ACT gene</td>
<td>Deshler <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>pICk2S</td>
<td>pICL20H with left Xba1/BamHI k2 fragment</td>
<td>P. A. Meacock</td>
</tr>
<tr>
<td>pGKF202</td>
<td>pBL3345 with right BamHI k2 fragment</td>
<td>Hishinuma <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>pRKL2</td>
<td>Linear k2ORF1a plasmid (ΔORF1 Δ:Δ ScLEU2)</td>
<td>Schaffrath <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pAR1</td>
<td>pUC19, for k1ORF2 disruption via ScLEU2 integration</td>
<td>Schaffrath <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pR51</td>
<td>pUC19 with k1UCS2-ScLEU2 marker gene fusion</td>
<td>Schaffrath <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pAR4</td>
<td>pUCBM21, as pAR1 for k1ORF2 disruption, but internal MCS; external HindIII converted into Nhel site</td>
<td>J. Schrönder &amp; F. Meinhardt, Münster, Germany</td>
</tr>
<tr>
<td>pSS9</td>
<td>pUC20H with PCR-engineered KI TRP I gene</td>
<td>S. M. Soond &amp; P. A. Meacock</td>
</tr>
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</table>

**DNA preparations, yeast plasmid mapping and Southern hybridization.** *E. coli* plasmid DNA was isolated by alkaline lysis (Sambrook *et al.*, 1989). Rapid preparations of *K. lactis* linear plasmid DNAs for screening transformants, and high-quality linear plasmid DNA preparations for restriction mapping and Southern blot analysis were performed as reported by Schaffrath & Meacock (1995). High-specific-activity labelling of DNA probes was carried out by random hexamer priming (Feinberg & Vogelstein, 1983) using [a-32P]dCTP (>3000 Ci mmol⁻¹; >111 TBq mmol⁻¹; Amersham). Radio-labelled probes were prepared from the following restriction fragments: KI ACT, 0.3 kb BglII from pACT3; KI TRP I, 0.7 kb NsiI/BamHI from pAR5; k1ORF2, 2.3 kb NsiI from pKLK109; k1ORF3, 0.6 kb BglII/EcoRI from pAR5; k1UCS2, 0.3 kb PstI/NsiI from pRS1; k2ORF2, 1.5 kb NsiI from pICk2S; k2ORF4, 1.7 kb BamHI/SnaBI from pGKF202; k2ORF6, 2.7 kb NsiI/Nhel from pGKF202 (see Table 2). For plasmid copy analysis, the strengths of k1UCS2 and k2ORF4 hybridization signals of recombinant plasmids, standardized to the values found for wild-type plasmids, were quantified using a Molecular Dynamics Phosphor-Imager.

**RNA isolation and Northern analysis.** Total RNA isolation and Northern analysis was performed as described by Schaffrath & Meacock (1995). DNA/RNA hybridization involved k2ORF2/ORF6 and ACT gene probes (see above).

**Plasmid functional analysis.** Plasmid segregation analysis was carried out by sequential Trp⁺ selective subcultivation of transformant strains over 350 generations in minimal SD medium supplemented with 40 μg leucine ml⁻¹, as reported by Schaffrath & Meacock (1995).

**RESULTS**

**Use of KI TRP I for selection of recombinant linear plasmids**

We wished to develop a second genetic marker that could be used for *in vivo* manipulation of the linear DNA killer plasmids of *K. lactis*. Thus, using an approach similar to previous work (Kämper *et al.*, 1991) the transfer vector pAR5 was constructed (Fig. 1). Plasmid pAR5 carries homologous k1 segments identical to pAR1 but employs the *K. lactis* TRP I gene fused in-phase to the UCS element of k1 ORF2 (k1 UCS2) rather than *S. cerevisiae* LEU2 (Table 2; Schaffrath *et al.*, 1992). This new marker gene is different in size to the previous ScLEU2-based marker allowing easy identification of recombinant linear plasmids. More significantly, these two markers can be used together in strain AWJ137, which carries chromosomal leu2 and trp1 mutations, for procedures requiring two independent manipulations of the plasmid system. It was therefore important to establish that the trp1 mutation of strain AWJ137 had a sufficiently low reversion frequency that recombinant linear plasmids could be selected and segregated by selection for k1 UCS2-KI TRP I marker in a similar manner to the ScLEU2 marker. Thus, DNA of plasmid pAR5, completely digested with Nhel and EcoRI, was used to transform the k1/k2-carrying strain AWJ137 to tryptophan prototrophy. Trp⁺ transformants were obtained at high
Table 3. Frequencies of k1 and k2 gene targeting

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Vector</th>
<th>Frequency [ng vector DNA]</th>
<th>Hybrid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1ORF2</td>
<td>pAR5</td>
<td>260–430</td>
<td>pRKL1(100)</td>
</tr>
<tr>
<td>k2ORF6</td>
<td>pMS600</td>
<td>230–290</td>
<td>rk2/6 (100)</td>
</tr>
<tr>
<td>k2ORF2</td>
<td>pMS202</td>
<td>140–170</td>
<td>rk2/2 (96)</td>
</tr>
</tbody>
</table>

*Percentage of transformants carrying hybrid plasmid is in parentheses.

Fig. 2. Demonstration of k1ORF2 disruption in plasmid pRKL1. Linear plasmid DNA preparations from wild-type strain AWJ137 (lane 2) and pRKL1-harbouring strain MS1 grown for 25, 50 and 150 g.p.r. (lanes 3–5) were separated on agarose gels and hybridized with gene probes as indicated. Migration positions of linear plasmids k1, k2 and pRKL1 are indicated by arrows. Molecular mass markers (lane 1) were the 1 kb ladder (Gibco, BRL).

predicted (Fig. 2). Strain MS1 was subjected to prolonged cultivation in the absence of tryptophan to select for cells carrying pRKL1. As shown in Fig. 2, segregation of parental k1 from the hybrid pRKL1 was observed, eventually leading to complete loss of k1 over a growth period of 150 generations post-recombination (g.p.r.). Hybridization with radiolabelled probes made from k2ORF6, the KITRP1 gene, and the k1-derived ORF2 and ORF3 (Fig. 2), produced no signals corresponding to parental k1; only pRKL1 and k2 were maintained in the segregant strain, termed NKMS1. Thus, pRKL1 was able to displace wild-type k1, indicating the TRP1 selection regime to be successful in conjunction with the genetic background of strain AWJ137.

Transcriptional analysis of k2ORF2 and k2ORF6
Total RNA from both plasmid-containing and plasmid-free K. lactis strains was subjected to Northern analysis using k2 DNA fragments from within the ORF2 and

Fig. 1. Maps of plasmid vectors pAR5, pM600 and pMS202 used for genetic manipulation of (a) k1ORF2, (b) k2ORF6 and (c) k2ORF2, respectively. Flanking sequences that promote homologous recombination in vivo into the native k1 or k2 linear plasmid are shown in cross-hatching. The remnant k1 and k2 ORF sequences are indicated by ~. The UCS–TRP1 fusion marker is denoted by an arrow preceded by an open box. For construction see Methods.

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<td>pMS600</td>
<td>230–290</td>
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<td>k2ORF2</td>
<td>pMS202</td>
<td>140–170</td>
<td>rk2/2 (96)</td>
</tr>
</tbody>
</table>

*Percentage of transformants carrying hybrid plasmid is in parentheses.
Yeast plasmid pGKL2 DNA and RNA polymerase genes

Fig. 3. Analysis of k2ORF6 transcription. Northern blot analysis of total RNA (20 µg per lane) extracted from the k1/k2-carrying strain AWJ137 (lane 1) and k1/k2-free strain SD801 (lane 2). Hybridization probes were the *K. lactis*-specific actin and ORF6 DNA fragments (see Methods). Arrowheads indicate positions of the 265 and 185 yeast rRNAs and sizes of co-migrated RNA markers are indicated. Positions of the actin and ORF6 transcripts are indicated.

Fig. 4. Demonstration of k2ORF6 disruption in plasmid rk2/6. Linear plasmid DNA preparations from wild-type strain AWJ137 (lane 3) and rk2/6-carrying strain MS60 grown for 150 and 350 g.p.r. (lanes 4–5) were separated on agarose gels and hybridized to gene probes as indicated. Strain MS1 carrying pRKL1 (lane 2) served as positive control for the k1UCS2–TRP1 gene. Migration positions of linear plasmids k1, k2, rk2/6 and pRKL1 are indicated by arrows. Molecular mass markers (lane 1) were the 1 kb ladder (Gibco, BRL).

ORF6 coding regions as hybridization probes. As shown in Fig. 3, this analysis revealed a single transcript for ORF6 of estimated size 3.1 kb which was undetectable in RNA from plasmid-free cells. For ORF2, only a weak hybridization signal was detected indicating a less abundant transcript of 3.2 kb (data not shown). Comparison of ORF6 and actin mRNA levels in exponential-phase cultures indicates an overall low cellular transcription of the putative RNA polymerase gene when relative gene copy levels, one for actin versus many for the plasmid-borne k2ORF6, are taken into account (Fig. 3).

Disruption of k2ORF6

The novel TRP1 marker described above was used to construct pMS600, a k2ORF6 disruption vector (Fig. 1b). In pMS600, the UCS–TRP1 fusion gene is bracketed by segments homologous to k2 which include short remnants of the 5' and 3' terminal regions of ORF6. Thus, allelic replacement of ORF6 by the TRP1 marker gene should occur following homologous recombination between native k2 and the disruption cartridge; this will result in formation of ORF6 deletion plasmids. Wild-type killer strain AWJ137 was transformed with a *HindIII*/NdeI digest of pMS600 and Trp⁺ clones were obtained at high frequency (Table 3). Plasmid analysis revealed that a new plasmid, termed rk2/6, was present in addition to k2 and k2 in all 48 independent isolates examined. Southern blot analysis of one representative transformant, designated as MS60, showed that in rk2/6 the TRP1 marker had replaced the putative RNA polymerase gene ORF6 (Fig. 4). Moreover, rk2/6 was identified as a k2-derivative by hybridization to the k2ORF2 probe and was found to carry the k1UCS2 element (Fig. 4). Long-term selection for TRP1 failed to favour establishment of hybrid rk2/6 over k2. Even after 350 g.p.r., strain MS60 was still found to contain both k2 and the recombinant plasmid (Fig. 4);

no apparent copy number changes could be detected as judged from intensities of plasmid bands on agarose gels. Thus, in contrast to the plasmid pair k1/pRKL1 (see above), plasmid segregation and displacement of k2 by rk2/6 was not detectable even though the same selection marker was used. Therefore, maintenance of the ORF6 deletion plasmid rk2/6 appeared to be strictly dependent on the presence of wild-type k2, indicating that the putative RNA polymerase target gene represents an essential locus for plasmid functionality.

Disruption of k2ORF2

In an approach similar to that for ORF6, we next constructed transfer vector pMS202 for disruption of the putative DNA polymerase gene ORF2 on k2. The UCS–TRP1 fusion gene of pMS202 is placed in opposite polarity to ORF2 and flanked by k2 segments including remnant 3' and 5' terminal fragments of ORF2 to promote recombination with parental k2 and formation of ORF2 deletion plasmids *in vivo*. Transformation of AWJ137, with *HindIII*-cut pMS202, to tryptophan prototrophy yielded Trp⁺ clones at high frequency (Table 3), and 46 out of 48 transformants analysed for plasmid patterns were found to carry a novel plasmid, termed rk2/2 (data not shown). Southern hybridization analyses confirmed the expected structure of rk2/2; positive hybridization was found using probes specific for k2ORF6, k1UCS2 and TRP1 (Fig. 5), whereas no hybridization to the central ORF2-spanning probe (Fig. 5) was detected. Thus, we concluded that rk2/2 had undergone the expected structural changes during recombination leading to ORF2
allelic replacement by the UCS-TRP1 marker. Prolonged cultivation under selective pressure of a representative transformant, designated MS20, revealed plasmid segregational behaviour comparable to that seen for ORF6 disruption; as is the case with rk2/6, the ORF2 deletion plasmid rk2/2 was unable to displace wild-type k2 under selective growth conditions. Again, relative copy numbers appeared to be unaffected at both 150 and 350 g.p.r., respectively (Fig. 5) with rk2/2 levels predominantly low compared to wild-type k2. Thus, we concluded that ORF2 encodes an essential function involved in plasmid replication and/or maintenance.

Physical characterization of hybrid k2 deletion plasmids

Plasmids rk2/2 and rk2/6 were isolated from strains MS20 and MS60, respectively, by electroelution of proteinase K-treated DNA samples. Restriction mappings confirmed that k2 had undergone the predicted in vivo structural changes that resulted in generation of hybrid plasmids rk2/2 and rk2/6 (data not shown). As summarized in Fig. 6, new SstI and BamHI sites were introduced into rk2/2 and rk2/6 by homologous recombination resulting in plasmid restriction patterns distinguishable from that of parental plasmid k2 and confirming the linear structure of both hybrids. Thus, both rk2/2 and rk2/6 are co-linear in genome organizations with k2 except for deletion of the ORF2 and ORF6 regions by replacement with the UCS-TRP1 marker gene. Both rk2/2 and rk2/6 have decreased sizes (12.3 and 11.8 kb, respectively) when compared to parental k2 (13.5 kb) (Fig. 6).

Relative plasmid copy number and stability

We examined whether the hybrid plasmids were less stably maintained when ORF2 and ORF6 had been deleted. The recombinant plasmids rk2/2 and rk2/6 in MS20 and MS60 had reduced stabilities in comparison to wild-type k2 in cultures grown without selection for tryptophan prototrophy. In each case, all 200 colonies obtained after sequentially subculturing in YPD rich medium for 350 generations, were found to be Trp+ as a result of having lost the hybrid plasmid rk2/2 or rk2/6 (data not shown). Thus, integration of TRP1 into ORF2 and ORF6 decreased the stability of the resulting recombinant plasmids. To assess relative plasmid copy numbers of the recombinant k2 plasmids versus wild-type k1 and k2 in cultures grown with Trp+ selection, we carried out Southern hybridization analysis using kl- and k2-derived gene probes and quantified the abundance of each plasmid with a Phosphor-Imager (Fig. 7). Plasmid copy numbers of rk2/2 and rk2/6 were significantly reduced in comparison to the k2ORF1 deletion plasmid pRKL2.
Yeast plasmid pGKL2 DNA and RNA polymerase genes

Fig. 7. Plasmid copy-number analysis. Linear plasmid DNA preparations from wild-type killer strain AWJ137 and various k2ORF-deletion strains were separated on agarose gels and hybridized to gene probes as indicated. Lanes: 1, molecular mass markers; 2-3, wild-type AWJ137; 4-5, k2ORF2 deletion strain MS20; 6-7, k2ORF1 deletion strain KRS13; 8-9, k2ORF6 deletion strain MS60. Growth for MS20, MS60 and KRS13 was 350 g.p.r. To determine the extent of hybridization of hybrid plasmids to the probes, relative band intensities were quantified using a Molecular Dynamics Phosphor-Imager and wild-type plasmids k1 and k2 from AWJ137 as internal controls (see Table 4). Migration positions of linear plasmids k1, k2, rk2/2, rk2/6 and pRKL2 are indicated by arrows.

(Schaffrath et al., 1992). Relative copy numbers were lower for hybrid rk2/2 than rk2/6, implying direct involvement of the ORF2 product in plasmid replication and an indirect maintenance function for ORF6 (Fig. 7). For the ORF6 mutant strain MS60, levels of all linear plasmids in the cell appeared to be generally affected, whereas the ORF2 deletion strain MS20 appeared to possess normal levels of wild-type k1 and k2 (Fig. 7). Table 4 summarizes the results from seven independent experiments using duplicate plasmid samples of AWJ137, MS20 and MS60 (data not shown); rk2/2 was found to have a sevenfold lower copy number relative to wild-type k2, whereas rk2/6 was reduced only fourfold compared to wild-type k2 copy numbers.

Table 4. Relative plasmid copy numbers

Determined by measurement of hybridization strength using a Molecular Dynamics Phosphor-Imager. In each strain, recombinant plasmids were monitored against copy levels of a wild-type plasmid carrying the same hybridization target sequence. Thus, the wild-type plasmid was assigned a value of 1 and the recombinant plasmid a copy level value proportional to the strength of the hybridization signal.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Hybridization probes</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>k1UCS2</td>
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<td>AWJ137</td>
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DISCUSSION

We report here the genetic analysis of ORF2 and ORF6 of the cytoplasmic linear killer plasmid k2 of K. lactis. Our data show these ORFs to be transcriptionally active genes essential for the linear plasmid system.

As with previous studies on the k1ORF2 and k2ORF1 deletion plasmids pJKL1 and pRKL2 (Käumper et al., 1991; Schaffrath et al., 1992), in vivo generation of hybrid plasmids pRKL1, rk2/2 and rk2/6 occurred at frequencies approaching 100%. Thus, plasmid recombination is a highly specific process unlike the high level of non-homologous recombination events seen for nuclear gene targeting in K. lactis (Stark & Milner, 1989; Meinhardt & Holtwick, 1995). Plasmid gene targeting at reproducibly high frequencies presumably reflects a copy-number-dependence comparable to recent recombination studies in S. cerevisiae (Wilson et al., 1994). Whether plasmid recombination is dependent on nuclear functions, such as those of the RAD genes, or takes place in the cytoplasm by an alternative mechanism, has to await further detailed studies and isolation of K. lactis RAD mutants as described for S. cerevisiae (Dunderdale & West, 1994).

Both k2ORF2 and ORF6 partially overlap with other upstream ORFs, ORF3 and ORF5, respectively, on the same DNA strand (Tommasino et al., 1988). However, Northern blot analysis revealed only single ORF2 and ORF6 transcripts of approximately 3.2 and 3.1 kb. Thus, both genes are probably transcribed as monocistronic units implying the existence of a mechanism that terminates ORF3 and ORF5 transcription while allowing ORF2 and ORF6 transcription to occur.

The novel UCS marker gene fusion used in this study, k1UCS2–TRP7, provides further substantial evidence for the existence of a plasmid-specific transcription system. Not only were we able to use this construct to generate the k1ORF2 deletion plasmid pRKL1, but we were also able to demonstrate its use in k2 gene disruptions. Thus, in vivo formation of the k2ORF2 and k2ORF6 deletion plasmids rk2/2 and rk2/6 could be successfully selected...
by use of the TRP1 marker gene. Our results together with previous studies on the k2ORF1 deletion plasmid pRKL2 (Schaffrath et al., 1992) show that a k1-derived UCS element is functionally active when placed onto k2. This is entirely consistent with the hypothesis that the conserved sequence motif, UCS, found upstream of all linear plasmid genes, represents a cytoplasmic promoter that is recognized by the putative k2-encoded RNA polymerase (Wilson & Meacock, 1988; Romanos & Boyd, 1988). Also consistent with this are recent reporter gene fusions showing the UCS5 motif of k2 to be an essential cis-acting element for foreign gene expression (Meinhardt et al., 1994; Schaffrath & Meacock, 1995).

Our data on successful plasmid segregation of pRKL1 from k1, resulting in pure recombinant pRKL1/k2 constellations as in segregant NKMS1, are very similar to results obtained with k1ORF2 and k2ORF1 deletion plasmids pJKL1 and pRKL2 (Kämper et al., 1991; Schaffrath et al., 1992). These derivatives were also able to displace their parental wild-type plasmids, k1 and k2, respectively. Plasmid segregation as a consequence of progressive copy number decrease of the wild-type plasmid versus the recombinant during selective growth implies competition between hybrid and parental plasmids for rate-limiting maintenance factors, perhaps the k1/k2 TPs, and an overall randomness in the choice of molecules for plasmid replication and/or partitioning.

In the cases of the ORF2- and ORF6-deletion plasmids rk2/2 and rk2/6, their failure to displace k2, even over a period of 350 generations of selective growth post-recombination, implies that both genes are required for plasmid functionality. This is a similar situation to that observed with k2ORF5 (Schaffrath & Meacock, 1995). The inability of rk2/2 to displace k2 is particularly interesting since these cells contained plasmid k1 which also encodes a putative DNA polymerase (k1ORF1). Therefore, we conclude that the k1ORF1-encoded DNA polymerase is unable to complement the defect arising by deletion of the k2ORF2-encoded DNA polymerase. We interpret this to mean that these two DNA polymerases are each specific for the plasmid that encodes them. Presumably this specificity resides in the recognition of the relevant TP/TIR structures for replication initiation. A similar interpretation can be drawn from the behaviour of pk129L/S, k1-hybrids deleted in the ORF1 putative k1 DNA polymerase structural gene (Fukuhara, 1987; Kitada & Gunge, 1988), which cannot be maintained without the presence of a fully functional k1ORF1 in the same cell despite the presence of k2.

Interestingly, relative plasmid copy levels of k2 and the recombinant plasmids remained unaffected during selective growth; rk2/2 had a lower copy level relative to wild-type k2 than did rk2/6. This may reflect the amount of each gene product necessary for the plasmid system to function. Thus, we propose direct involvement of the ORF2 product in k2 replication and an indirect k1/k2 maintenance function for the putative ORF6-encoded RNA polymerase. This provides further evidence of the fundamental role played by k2 in the integrity and maintenance of the overall killer plasmid system.

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