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

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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 *KITR1* 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; Gunge *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* Tn903-derived G418^R, were transplaced 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

Abbreviations: g.p.r., generations post-recombination; k1, pGKL1; k2, pGKL2; mcs, multiple cloning site; TIR, terminal inverted repeat; TP, terminal protein; UCS, upstream conserved sequence.

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-*TRP1* 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-TAGGTAAAGTAAC 3' (k2ORF6); and k2 pos. 11280–11299, FW7 5' CATATGAATGAAAATATTATTTC 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. Schröder & F. Meinhardt, personal communication) was replaced by the 0.7 kb *NsiI/BamHI* *K1TRP1* gene fragment of pSS9 (S. M. Soond & P. A. Meacock, unpublished). For convenience, the external *pUC*-based *HindIII* site was converted into an *NheI* site by restriction,

Klenow fill-in and re-ligation (Fig. 1a). Thus, digestion of pAR5 with *NheI* 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 *NcoI* k2 fragment, obtained from pRS23, a k2 subclone derived from pGKF202, was cloned into the single *NcoI* 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 *SmaI*-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 *K1TRP1* gene from pAR5. Thus, in pMS600 the k1UCS2 element is fused in-phase to *TRP1*. 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 pRS36, 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 *K1TRP1* gene from pAR5 as outlined above. Part of the ampicillin resistance gene of pRF2 was released upon *SmaI/ScaI* double restriction and functionally reconstituted with the 3.3 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 *TRP1* marker gene (Fig. 1c).

Table 1. Strains

Strain	Description	Source
<i>E. coli</i>		
NM522	$\Delta(lac-proAB) thi supE \Delta(bsdMS-mcrB)5 r_K^- m_K^- mcrB/F' (proAB^+ lacI^a lacZ \Delta M15)$	Gough & Murray (1983)
<i>K. lactis</i>		
AWJ137	$\alpha len2 trp1 (k1^+ k2^+)$ (killer, immune)	Kämper <i>et al.</i> (1991)
SD801	$\alpha trp1 lac4 (k1^0 k2^0)$ (non-killer, sensitive)	Stark & Milner (1989)
KRS13	$\alpha len2 trp1 [k1^+ pRKL2 (k2\Delta ORF1::k1UCS2 ScLEU2)]$ (killer, immune)	Schaffrath <i>et al.</i> (1992)
MS1	$\alpha len2 trp1 [pRKL1 (k1\Delta ORF2::K1TRP1) k1^+ k2^+]$ (killer, immune)	This work
NKMS1	$\alpha len2 trp1 [pRKL1 k2^+]$ (non-killer, immune)	This work
MS20	$\alpha len2 trp1 [k1^+ k2^+ rk2/2 (k2\Delta ORF2::k1UCS2 K1TRP1)]$ (killer, immune)	This work
MS60	$\alpha len2 trp1 [k1^+ k2^+ rk2/6 (k2\Delta ORF6::k1UCS2 K1TRP1)]$ (killer, immune)	This work

Table 2. Plasmids

Plasmid	Description	Source
pUC13/BM21	General cloning vectors	Pharmacia/Boehringer
k1/k2	Linear killer plasmids (8.9/13.5 kb) from <i>K. lactis</i>	Gunge <i>et al.</i> (1981)
pK1K109	pUC9 with internal k1 fragment	Stark <i>et al.</i> (1984)
pACT3	pUC9 with <i>K1ACT</i> gene	Deshler <i>et al.</i> (1989)
pICk2S	pIC20H with left <i>XbaI/BamHI</i> k2 fragment	P. A. Meacock
pGKF202	pBLS345 with right <i>BamHI</i> k2 fragment	Hishinuma <i>et al.</i> (1984)
pRKL2	Linear k2ORF1 ⁰ plasmid (Δ ORF1::k1UCS2 <i>ScLEU2</i>)	Schaffrath <i>et al.</i> (1992)
pAR1	pUC19, for k1ORF2 disruption <i>via ScLEU2</i> integration	Schaffrath <i>et al.</i> (1992)
pRS1	pUC19 with k1UCS2– <i>ScLEU2</i> marker gene fusion	Schaffrath <i>et al.</i> (1992)
pAR4	pUCBM21, as pAR1 for k1ORF2 disruption, but internal MCS; external <i>HindIII</i> converted into <i>NheI</i> site	J. Schröder & F. Meinhardt, Münster, Germany
pSS9	pIC20H with PCR-engineered <i>K1TRP1</i> gene	S. M. Soond & P. A. Meacock
pAR5	As pAR4, but 1.6 kb <i>ScLEU2</i> replaced by 0.7 kb <i>K1TRP1</i>	This work
pRKL1	Linear k1ORF2 ⁰ plasmid (Δ ORF2::K1TRP1)	
pRS23	pUCBM21 with k2 insert of pGKF202 (pos. 7314–11008)	
pRS27	pUCBM21 with 0.8 kb k2ORF5-spanning fragment from pRS23	
pRS16	pUC13 with 0.6 kb k2ORF7 PCR fragment	
pRS36	pRS27 with k1UCS2 <i>LEU2</i> fusion marker from pRS1	
pMS60	pRS36 with second k2ORF7-spanning fragment of pRS16	
pMS600	pMS60 with <i>ScLEU2</i> replaced by <i>K1TRP1</i> from pAR5	
rk2/6	Linear k2ORF6 ⁰ plasmid (Δ ORF6::k1UCS2K1TRP1)	
pRF1	pRS36 with k2ORF5 replaced by k2ORF1 from pICk2S	
pRF2	pRF1 with <i>ScLEU2</i> replaced by <i>K1TRP1</i> from pAR5	
pMS202	pRF2 with second k2ORF2/3-spanning fragment from pICk2S	
rk2/2	Linear k2ORF2 ⁰ plasmid (Δ ORF2::k1UCS2K1TRP1)	

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 [α -³²P]dCTP (> 3000 Ci mmol⁻¹; > 111 TBq mmol⁻¹; Amersham). Radio-labelled probes were prepared from the following restriction fragments: *K1ACT*, 0.3 kb *BglII* from pACT3; *K1TRP1*, 0.7 kb *NsiI/BamHI* from pAR5; k1ORF2, 2.3 kb *NsiI* from pK1K109; 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 *NcoI/NdeI* 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 μ g leucine ml⁻¹, as reported by Schaffrath & Meacock (1995).

RESULTS

Use of *K1TRP1* 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 TRP1* gene fused in-phase to the UCS element of k1ORF2 (k1UCS2) 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 *len2* 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 k1UCS2–*K1TRP1* marker in a similar manner to the *ScLEU2* marker. Thus, DNA of plasmid pAR5, completely digested with *NheI* and *EcoRI*, was used to transform the k1/k2-carrying strain AWJ137 to tryptophan prototrophy. Trp⁺ transformants were obtained at high

Fig. 1. Maps of plasmid vectors pAR5, pMS600 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 Δ . The UCS-TRP1 fusion marker is denoted by an arrow preceded by an open box. For construction see Methods.

frequencies (Table 3) and 48 randomly selected clones were all found to carry a new plasmid, termed pRKL1, in addition to k1 and k2. Southern blot hybridization analysis of one representative clone, MS1, revealed pRKL1 to have ORF2 replaced by the TRP1 gene as

Table 3. Frequencies of k1 and k2 gene targeting

Target gene	Vector	Frequency [(μ g vector DNA) ⁻¹]	Hybrid*
k1ORF2	pAR5	260–430	pRKL1(100)
k2ORF6	pMS600	230–290	rk2/6 (100)
k2ORF2	pMS202	140–170	rk2/2 (96)

* 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-harboring 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 K1TRP1 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. 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 26S and 18S yeast rRNAs and sizes of co-migrated RNA markers are indicated. Positions of the actin and ORF6 transcripts are indicated.

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 ORF6⁰ deletion plasmids. Wild-type killer strain AWJ137 was transformed with a *Hind*III/*Nde*I 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 k1 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);

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 pRK1 (lane 2) served as positive control for the k1UCS2-*TRP1* gene. Migration positions of linear plasmids k1, k2, rk2/6 and pRK1 are indicated by arrows. Molecular mass markers (lane 1), were the 1 kb ladder (Gibco, BRL).

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/pRK1 (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 ORF6⁰ 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 *Hind*III-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

Fig. 5. Demonstration of k2ORF2 disruption in plasmid rk2/2. Linear plasmid DNA preparations from wild-type strain AWJ137 (lane 3) and rk2/2-carrying strain MS20 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 pRK1 (lane 2) served as positive control for the k1UCS2–*TRP1* gene. Migration positions of linear plasmids k1, k2, rk2/2 and pRK1 are indicated by arrows. Molecular mass markers (lane 1) were the 1 kb ladder (Gibco, BRL).

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 *Sst*I and *Bam*HI 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

Fig. 6. Co-linearity of hybrid plasmids rk2/2 and rk2/6 with parental k2. The physical maps shown for rk2/2, rk2/6 and k2 were obtained from Southern hybridization analyses of restriction digests involving electro-eluted DNA substrates and radioactive probes as follows: (a) k1UCS2, (b) K1*TRP1*, (c) k2ORF6 and (d) k2ORF2. Restriction enzyme cleavage patterns are given below each map. Numbering of ORFs and k2, rk2/2 and rk2/6 genome nomenclature are adopted from Tommasino *et al.* (1988); positions and polarities of ORFs and the *TRP1* gene are indicated by open arrows. The k1UCS2 element driving *TRP1* is indicated by a black bar. TIRs and TPs of the plasmids are presented as black triangles and black circles, respectively. The remainders of ORF2 in rk2/2 and ORF6 in rk2/6 are indicated by Δ .

(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 k1- 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 pRK12

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.

Strain	Plasmid	Hybridization probes	
		k1UCS2	k2ORF4
AWJ137	k1	1	
	k2		1
MS60	k1	1	
	k2		1
MS20	rk2/6	0.25	0.24
	k1	1	
	k2		1
	rk2/2	0.13	0.15

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 (Kämper *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-*TRP1*, 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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REFERENCES

- Bolen, P. L., Eastman, E. M., Cihak, P. L. & Hayman, G. T. (1994). Isolation and sequence analysis of a gene from the linear DNA plasmid pPar1-2 of *Pichia acaciae* that shows similarity to a killer toxin gene of *Kluyveromyces lactis*. *Yeast* **10**, 403–414.
- Cong, Y.-S., Yarrow, D., Li, Y.-Y. & Fukuhara, H. (1994). Linear DNA plasmids from *Pichia etchellsii*, *Debaryomyces hansenii* and *Wingea robertsiae*. *Microbiology* **140**, 1327–1335.
- Deshler, J. O., Larson, G. P. & Rossi, J. J. (1989). *Kluyveromyces lactis* maintains *Saccharomyces cerevisiae* intron-encoded splicing signals. *Mol Cell Biol* **9**, 2208–2213.
- Dunderdale, H. J. & West, S. C. (1994). Recombination genes and proteins. *Curr Opin Genet Dev* **4**, 221–228.
- Feinberg, A. P. & Vogelstein, B. (1983). A technique for radio-labeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**, 6–13.
- Fukuhara, H. (1987). The RF1 gene of the killer DNA of yeast may encode a DNA polymerase. *Nucleic Acids Res* **15**, 10046.
- Gietz, D., St Jean, A., Woods, R. A. & Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**, 1425.
- Gough, J. A. & Murray, N. E. (1983). Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J Mol Biol* **166**, 1–19.
- Gunge, N., Tamaru, A., Ozawa, F. & Sakaguchi, K. (1981). Isolation and characterization of linear deoxyribonucleic acid plasmids from *Kluyveromyces lactis* and the plasmid-associated killer character. *J Bacteriol* **145**, 382–390.
- Gunge, N., Fukuda, K., Morikawa, S., Murakami, K., Takeda, M. & Miwa, A. (1993). Osmophilic linear plasmids from the salt-tolerant yeast *Debaryomyces hansenii*. *Curr Genet* **23**, 443–449.
- Hishinuma, F. & Hirai, K. (1991). Genome organisation of the linear plasmid pSKL isolated from *Saccharomyces kluyveri*. *Mol & Gen Genet* **226**, 97–106.
- Hishinuma, F., Nakamura, K., Hirai, K., Nishizawa, R., Gunge, N. & Maeda, T. (1984). Cloning and nucleic acid sequences of the linear DNA plasmids from yeast. *Nucleic Acids Res* **12**, 7581–7597.
- Kämper, J., Esser, K., Gunge, N. & Meinhardt, F. (1991). Heterologous expression on the linear DNA killer plasmid from *Kluyveromyces lactis*. *Curr Genet* **19**, 109–118.
- Kitada, K. & Gunge, N. (1988). Palindrome-hairpin linear plasmids possessing only part of the ORF1 gene of the yeast killer plasmid pGKL1. *Mol & Gen Genet* **215**, 46–52.
- McNeel, D. G. & Tamanoi, F. (1991). Terminal region recognition factor 1, a DNA binding protein recognizing the ITRs of the pGKL linear plasmids. *Proc Natl Acad Sci USA* **88**, 11398–11402.
- Meinhardt, F. & Holtwick, R. (1995). Generation of a stable non-reverting Leu⁻ mutant of *Kluyveromyces lactis* by gene disruption. *Appl Microbiol Biotechnol* **42**, 734–737.
- Meinhardt, F. & Rohe, M. (1993). Extranuclear inheritance: linear protein-primed replicating genomes in plants and microorganisms.

In *Progress in Botany*, vol. 54, pp. 334–357. Edited by H. D. Behnke, K. Esser, K. Kubitzki, M. Runge & H. Ziegler. Berlin, Heidelberg & New York: Springer-Verlag.

Meinhardt, F., Kempken, F., Kämpfer, J. & Esser, K. (1990). Linear plasmids among eukaryotes: fundamentals and application. *Curr Genet* **17**, 89–95.

Meinhardt, F., Wodara, C., Larsen, M. & Schickel, J. (1994). A novel approach to express a heterologous gene on *Kluyveromyces lactis* linear killer plasmids: expression of the bacterial *aph* gene from a cytoplasmic promoter fragment without in-phase fusion to the plasmid open reading frame. *Plasmid* **32**, 318–327.

Rohe, M., Schröder, J., Tudzinsky, P. & Meinhardt, F. (1992). Phylogenetic relationships of linear, protein-primed replicating genomes. *Curr Genet* **21**, 173–176.

Romanos, M. & Boyd, A. (1988). A transcriptional barrier to expression of cloned toxin genes of the linear plasmid k1 of *Kluyveromyces lactis*: evidence that native k1 has novel promoters. *Nucleic Acids Res* **16**, 7333–7350.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Schaffrath, R. & Meacock, P. A. (1995). *Kluyveromyces lactis* killer plasmid pGKL2: molecular analysis of an essential gene, ORF5. *Yeast* **11**, 615–628.

Schaffrath, R., Stark, M. J. R., Gunge, N. & Meinhardt, F. (1992). *Kluyveromyces lactis* killer system: ORF1 of pGKL2 has no function in immunity expression and is dispensable for killer plasmid replication and maintenance. *Curr Genet* **21**, 357–363.

Schaffrath, R., Soond, S. M. & Meacock, P. A. (1995). Cytoplasmic gene expression in yeast: a plasmid-encoded transcription system in *Kluyveromyces lactis*. *Biochem Soc Trans* **23**, 128S.

Sherman, F. (1991). Getting started with yeast. *Methods Enzymol* **194**, 3–21.

Stark, M. J. R. & Boyd, A. (1986). The killer toxin of *Kluyveromyces lactis*: characterization of the toxin subunits and identification of the genes which encode them. *EMBO J* **5**, 1995–2002.

Stark, M. J. R. & Milner, J. S. (1989). Cloning and analysis of the *Kluyveromyces lactis* TRP1 gene: a chromosomal locus flanked by genes encoding inorganic pyrophosphatase and histone H3. *Yeast* **5**, 35–50.

Stark, M. J. R., Boyd, A., Mileham, A. J. & Romanos, M. A. (1990). The plasmid-encoded killer system of *Kluyveromyces lactis*: a review. *Yeast* **6**, 1–29.

Tanguy-Rougeau, C., Chen, X. J., Wesolowski-Louvel, M. & Fukuhara, H. (1990). Expression of a foreign Km^R gene in a linear killer DNA plasmid in yeast. *Gene* **91**, 43–50.

Tokunaga, T., Wada, N. & Hishinuma, F. (1987). Expression and identification of immunity determinants on the linear DNA killer plasmids pGKL1 and pGKL2 in *Kluyveromyces lactis*. *Nucleic Acids Res* **15**, 1031–1046.

Tommasino, M. (1991). Killer system of *Kluyveromyces lactis*: the open reading frame 10 of the pGKL2 plasmid encodes a putative DNA binding protein. *Yeast* **7**, 245–252.

Tommasino, M., Ricci, S. & Galeotti, C. (1988). Genome organisation of the plasmid pGKL2 from *Kluyveromyces lactis*. *Nucleic Acids Res* **16**, 5863–5878.

Wilson, D. W. & Meacock, P. A. (1988). Extranuclear gene expression in yeast: evidence for a plasmid-encoded RNA polymerase of unique structure. *Nucleic Acids Res* **16**, 8097–8112.

Wilson, J. H., Leung, W. Y., Bosco, G., Dieu, D. & Haber, J. E. (1994). The frequency of gene targeting in yeast depends on the number of target copies. *Proc Natl Acad Sci USA* **91**, 177–181.

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