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Active recombination of pKD1-derived vectors with resident pKD1 in *Kluyveromyces lactis* transformation

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Summary. The host specificity of the 2 μ-like circular plasmid pKD1 is such that this plasmid replicates stably in several species of *Kluyveromyces* yeasts, but not in *Saccharomyces cerevisiae.* pKD1-derived plasmids containing various parts of the pKD1 sequence were capable of transforming *Kluyveromyces lactis* with high efficiency. When such vectors were introduced into host strains that contained resident pKD1 plasmid, the input DNA frequently recombined with it to produce high proportions of additive recombinant molecules that replicate stably. Recombination events were shown to occur with vectors differing for the presence or absence of the putative origin of replication and of the inverted repeats. Structure, stability and copy number of the recombination products were analyzed for various types of vectors.

Key words: *Kluyveromyces lactis* – Transformation – pKD1 – Recombination

Introduction

The efficient and stable transformation of *Kluyveromyces lactis* has recently been achieved by means of vectors based on the circular plasmid pKD1. This plasmid, which was originally isolated from *Kluyveromyces drosophilarum,* is also able to replicate in other *Kluyveromyces* species as well (Falcone et al. 1986; Chen et al. 1986; Bianchi et al. 1987). Its functional organization is analogous to that of the 2 μ plasmid of *Saccharomyces cerevisiae* (Hartley and Donelson 1980) and those of other circular plasmids found in osmotolerant *Zygosaccharomyces* (Araki et al. 1985; Toh-e and Utatsu 1985), even though there is little nucleotide sequence homology.

The vectors achieve the transformation of *K. lactis* with an efficiency comparable to that of the 2 μ system provided that they contain a 375 base-pair (bp) long segment of pKD1 adjacent to one of the inverted repeats (IR). This segment probably contains the replication origin (Bianchi et al. 1987).

In this paper we report a detailed analysis of the recombination events taking place between pKD1-derived transforming plasmids and resident pKD1 molecules. The proportion of the recombinant molecules was found to be higher than that observed in the 2 μ-*S. cerevisiae* system (Gerbaud et al. 1979; McNeil et al. 1980; Thomas and James 1980). Recombination not involving the IR sequence was also found to occur. These recombination events have important consequences for the control of plasmid maintenance, as well as in the performance of genetic transformation in this yeast.

Materials and methods

Yeast and bacterial strains. K. lactis strains, VD1 (pKD1°) and MD2/1 (pKD1$^+$) were used as hosts. These strains were obtained from strain MW98-8C (α, *uraA, argA, lysA,* K^+, *pKD1*°) (Bianchi et al. 1987). *Escherichia coli* transformation hosts were HB101 (*recA*$^-$) and RR1 (*recA*$^+$).

Plasmid construction and yeast transformation. The procedure for the construction of pKD1-derived plasmids (Fig. 1) and for the transformation of *K. lactis* have been described previously (Chen et al. 1986; Bianchi et al. 1987).

Media for yeast culture. The rich medium contained 2% glucose, 1% Difco Yeast Extract and 1% Difco Bacto Peptone. The selective medium contained 2% glucose and 0.7% Difco Yeast Nitrogen Base without amino acids supplemented with 10 μg/ml lysine and arginine.

Small-scale yeast DNA preparation. Yeast DNA was prepared from 5-ml rich medium cultures as described in Cameron et al. (1977).

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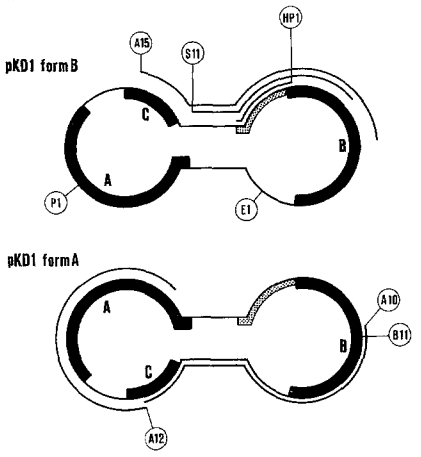

Fig. 1. Construction of pKD1-derived plasmids. The plasmid pKD1 is represented in two isomeric forms *B* (*top*) and *A* (*bottom*). Genes *A*, *B* and *C* are indicated by *thick lines*. The origin of replication has been localized within the *dotted block* (Bianchi et al. 1987). Gene *A* specifies the FLP recombinase function. pE1, pP1 and pB11 plasmids were obtained by inserting YIp5 into pKD1 at the unique sites EcoRI, PstI and ClaI, respectively, as indicated by *circled E1, P1* and *B11* symbols. Outer *thin lines*, marked by *circled A15, S11, HP1, A12* and *A10* symbols, delimit the portions of the pKD1 genome present in the corresponding plasmid constructs of the same name. *A15, A10* and *A12* are BamHI fragments and *S11* is a Sau3A fragment inserted in the BamHI site of YIp5; *HP1* is a HinPI fragment inserted into ClaI site of YIp5

Direct detection of recombinant plasmid molecules. Each transforming vector contained the complete YIp5 sequence (pBR322 plus the *URA3* gene from *S. cerevisiae*, which can complement the *uraA* mutation of *K. lactis*). This sequence has a single HindIII site, while the pKD1 DNA has none. When the resident pKD1 is integrated into the transforming DNA, the product can be linearized by HindIII, yielding a recombinant molecule that is 4,757 bp (length of the pKD1 sequence) larger than the input DNA. The input DNA and its recombinant molecules were selectively detected by Southern hybridization using a ^{32}P-labeled pBR322 probe. DNAs extracted from single yeast transformants as described above were digested with HindIII, electrophoresed on 0.6% agarose gels and blotted onto Hybond-N membrane (Amersham) according to the procedure suggested by the suppliers. After hybridization with radioactive pBR322 DNA, the signals of the input DNAs and their recombinant forms were detected by autoradiogrphy, and their relative intensities were determined.

Structure analysis of recombinant molecules. To analyze further the recombinant molecules, DNA from single yeast transformant clones was extracted as described above and used to transform *E. coli* HB101 and/or RR1 (Maniatis et al. 1982). The tetracycline or ampicillin resistance markers carried by the vectors were used for selecting the bacterial transformants. The plasmid population present in the original yeast clone was therefore represented in a series of *E. coli* transformants. Plasmid DNAs were extracted from many *E. coli* transformants (Maniatis et al. 1982) and analyzed by BamHI digestion followed by agarose gel electrophoresis. BamHI digestion of the pKD1 plasmid produced three fragments; 1,831, 1,498 and 1,428 bp (form A) or 1,717, 1,542 and 1,498 bp (form B). Recombination events between the resident pKD1 and the transforming DNA was revealed by the BamHI restriction pattern.

Stability of the K. lactis transformants. The transformants grown on selective plates were suspended in 5 ml rich liquid medium. An aliquot of the suspension was then diluted and plated on both complete and selective plates to determine the initial cell concentration and the percentage of *Ura*$^+$ cells (*s*). The culture was grown overnight (7–8 generations) at 28°C, to a concentration of 0.5–1 × 10^8 cells/ml, diluted and plated as described above to determine the final cell concentration and percentage of *Ura*$^+$ cells (*f*). The actual number of generations and the stability of the transformants (*100f/s*) were then calculated.

Plasmid copy number determination. Aliquots of the small-scale yeast DNA preparation were digested with HindIII, electrophoresed on 0.6% agarose gels and blotted onto Hybond-N membranes (Amersham) together with increasing amounts of HindIII-digested pBR322 DNA (from 0.025 ng to 6.4 ng) which was used as a quantitative reference scale. After hybridization with the radioactive pBR322 probe, the intensities of the signals were determined by comparing them to the reference scale, and the plasmid copy numbers per *Ura*$^+$cell were then calculated. In the *pKD1*$^+$strains, the number of resident pKD1 molecules was not affected by the presence of high- or low copy number vectors in the transformed cells, as deduced from the intensities of the pKD1 signals after ethidium bromide staining of the agarose gels. This number was estimated to be in the range of 50–70 copies per cell.

Results

Transformation of pKD1$^+$ *hosts with different classes of vectors, and the detection of recombinant molecules*

We observed previously (Bianchi et al. 1987) that the plasmid pA10, which lacks the origin of replication, is capable of producing transformant colonies of a normal size at high frequency (about 10^4/0.2 μg DNA). This was seen only when *pKD1*$^+$ hosts were used; no transformants were obtained with *pKD1*° hosts. This transformation results from the formation of additive recombinant molecules between plasmid pA10 and the resident pKD1, a recombination that may involve the inverted repeat sequence present in the transforming plasmid (Bianchi et al. 1987). The present results show that the stable transformation of *pKD1*$^+$ strains can also be achieved by means of a vector lacking both the replication origin and the IRs (pA12, see Fig. 1). When this construct was used for transformation, many very small, but visible, colonies were produced on selective plates. When these colonies were restreaked individually on fresh selective plates, none of them were able to continue growing when pKD1° was the host. However, when *pKD1*$^+$ hosts were used, the small colonies grew to a normal size after being restreaked. The transformants of the *pKD1*$^+$ hosts were highly stable, as in the case of the pA10 transformants mentioned above.

To analyze the events leading to transformation by ori$^-$ vectors, plasmid DNA extracted from some pA12 and pA10 individual, newly constructed transformants was digested with HindIII nuclease and analyzed by

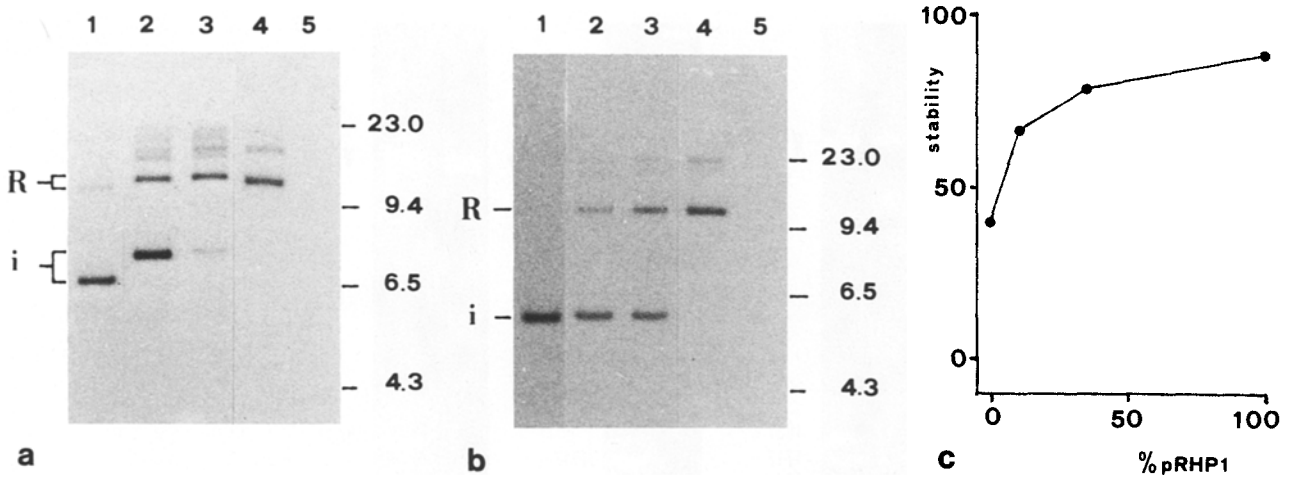

Fig. 2a–c. Detection of recombinant vectors by Southern analysis. **a** Analysis of DNAs from *K. lactis* transformants. *Lane 1,* pS11; *lane 2,* pA15; *lane 3,* pA10; *lane 4,* pA12; *lane 5,* untransformed host DNA (MD2/1 strain). **b** Analysis of a pHP1 *K. lactis* transformant. *Lane 1,* a newly constructed pHP1 transformant; *lanes 2, 3* and *4,* the same transformant after restreakings on selective plates; *lane 5,* host strain DNA. Molecular weight scales (kbp) are reported on right sides; *i* indicates the input vectors; *R,* the recombinant ones. *Upper signals* correspond to different combinations of vectors and pKD1 molecules. **c** Relationship between the stability of the *Ura*$^+$ phenotype and the percentage of pRHP1 present in a pHP1 transformant. The stability was determined on the same cell culture from which the DNA was extracted for Southern analysis

the Southern procedure (see Materials and methods). Figure 2a, lanes 3 (pA10) and 4 (pA12), show a very high proportion (84% for pA10 and 100% for pA12) of molecules that are either 4,757 bp longer than the input vectors (pRA10 and pRA12) or contain more than one integrated copy of resident pKD1. In these two cases, the high stability of the transformants (measured as the maintenance of the Ura$^+$ phenotype) resulted from the recombination of the vector with resident pKD1.

The same analysis was then extended to vectors containing both the origin of replication and the IRs (pS11 and pA15). This analysis (Fig. 2a, lanes 1 and 2) revealed the presence of signals corresponding to both the input vectors and the recombinant forms (pRS11 and pRA15). The proportion of recombinant molecules was much lower in the ori$^+$–IR$^+$ transformants than in the ori$^-$–IR$^{+/-}$ transformants.

A further analysis was performed with the pHP1 (ori$^+$–IR$^-$) transformants: only the unrecombined pHP1 vector could be detected in newly constructed pHP1 transformants (Fig. 2b, lane 1), while the same analysis revealed both unrecombined pHP1 and recombinant pRHP1 when the pHP1 transformants were subcultured many times by restreaking the clones on selective plates (Fig. 2b, lanes 2, 3 and 4). Southern analysis of restreaked pA12, pA10, pS11 and pA15 transformants did not reveal any variation in the proportion of recombinant molecules, while the proportion of pRHP1 molecules in the restreaked transformants increased at each transfer (see also Stability section and Fig. 2c).

Table 1. Proportion of recombinant molecules in the plasmid population of *K. lactis* transformants (*pKD1*$^+$)

Input DNA	Transformation frequency[a]	Percentage of recombinant molecules[c]
ori$^-$–IR$^-$		
pA12	[b]	100
ori$^-$–IR$^+$		
pA10	12,200	84 (± 4.7)
ori$^+$–IR$^+$		
pS11	11,200	13 (± 4.3)
pA15	6,600	33 (± 4.3)
ori$^+$–IR$^-$		
pHP1	8,800	0
pHP1[d]	8,800	Variable[e]

[a] Number of *Ura*$^+$ transformants/0.2 µg DNA per 3×10^6 protoplasts

[b] Thousands of visible, very small colonies were obtained which, after transfer to fresh minimal plates, grew normally. DNAs were extracted from these colonies for analysis

[c] Values are averages of three or four independent *K. lactis* transformants. Standard deviations are indicated in parentheses

[d] pHP1 transformants after repeated subculturing on selective medium plates

[e] See text and Fig. 2 for details

Results concerning the proportion of recombinant molecules determined by Southern analysis of newly constructed transformants obtained by different classes of vectors are reported in Table 1. Three or four

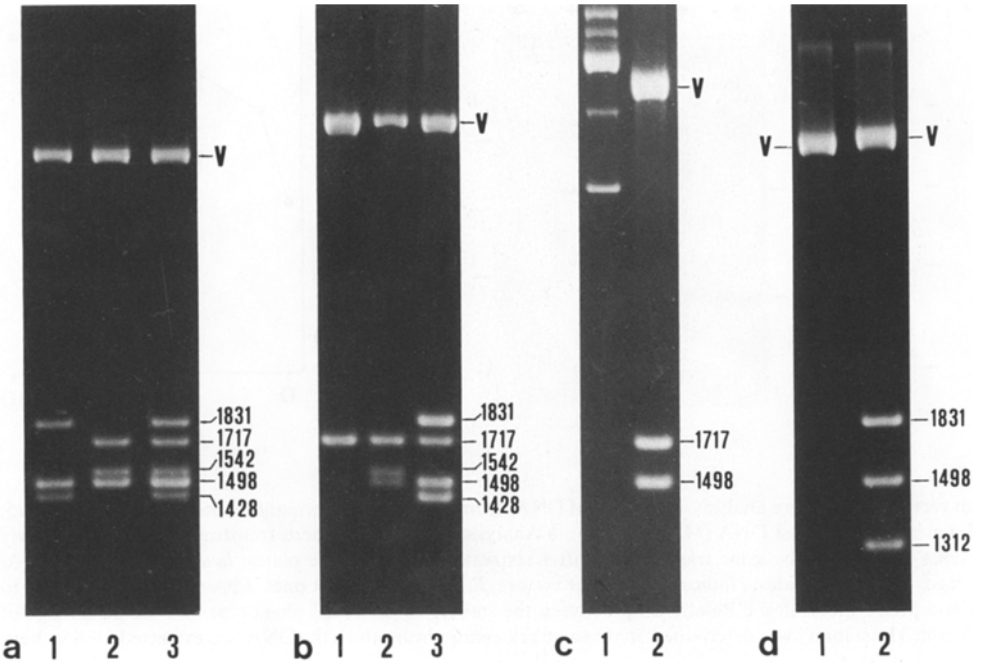

Fig. 3a–d. BamHI digestions of integrative recombinant plasmids. The plasmids were extracted from individual yeast transformant colonies, amplified in *E. coli,* then digested with BamHI. After gel electrophoresis, DNA bands were visualized by ethidium bromide staining. Note that some bands have double or triple intensity. **a** Recombination of pA12 with resident pKD1. *Lane 1* recombinant pRA12 (form A) shows 3 fragments of pKD1 form A and an entire YIp5 sequence (marked *v*); *lane 2* recombinant pRA12 (form B) shows 3 fragments of pKD1 form B and the YIp5 sequence (*v*); *lane 3* recombinant pRRA12 shows 5 fragments of both pKD1 isomer forms and the YIp5 sequence (*v*). **b** Recombination of pA15 and resident pKD1. *Lane 1* pA15 plasmid; *lane 2* pRA15 contains pKD1 form B fragments and YIp5; *lane 3* pRRA15 contains pKD1 fragments of both isomer forms and YIp5. **c** Recombination of pS11 with resident pKD1. *Lane 1* pS11. This construct has no BamHI sites and shows different circular forms; *lane 2* pRS11 shows two bands from pKD1 form B and a fragment containing YIp5 (*v*). **d** Recombination of pHP1 with resident pKD1. *Lane 1* pHP1 has a single BamHI site; *lane 2* pRHP1 shows 2 fragments of pKD1 form A, one junction fragment and one containing YIp5 (*v*)

independent transformants were analyzed for each vector. Very limited clonal variation was observed in each case.

Characterization of the recombinant molecules

To investigate the structure of these recombinant molecules, the DNAs extracted from the yeast transformants were subsequently used to transform *E. coli* so that the input vector and possible recombinant forms within a single yeast clone could be individually recovered in sets of bacterial transformants. One *K. lactis* transformant for each vector and three independent pA12 transformants were analyzed. The results are summarized in Table 2, where the number of the analyzed *E. coli* transformants is reported together with the numbers and characteristics of the retrieved vectors. These results are in agreement with those obtained by the Southern analysis; no significant differences were observed between newly constructed and subcultured yeast transformants, except in the case of pHP1 transformants.

Figure 3a shows examples of the restriction analysis performed on DNA from three different *E. coli* clones obtained from a pA12 yeast transformant. In lane 1, the double intensity of the 1,498 BamHI fragment and the presence of all the fragments expected for a BamHI digest of pKD1 (form A) indicated an additive recombination between pA12 and the resident pKD1 (pRA12), as diagrammed in Fig. 4a. A BamHI digestion of a similar recombination product containing pKD1 in form B is shown in Fig. 3a, lane 2. DNA in lane 3 contained all the fragments derived from forms A and B plus A12 with a triple intensity for the BamHI 1,498 band. This pattern showed that two molecules of pKD1 were integrated into pA12 (pRRA12). A similar analysis on pA10 transformants has been reported previously (Bianchi et al. 1987).

Recombinant molecules were also recovered after *E. coli* transformation (Table 2) with plasmid DNA obtained from yeast clones transformed with the ori$^+$–

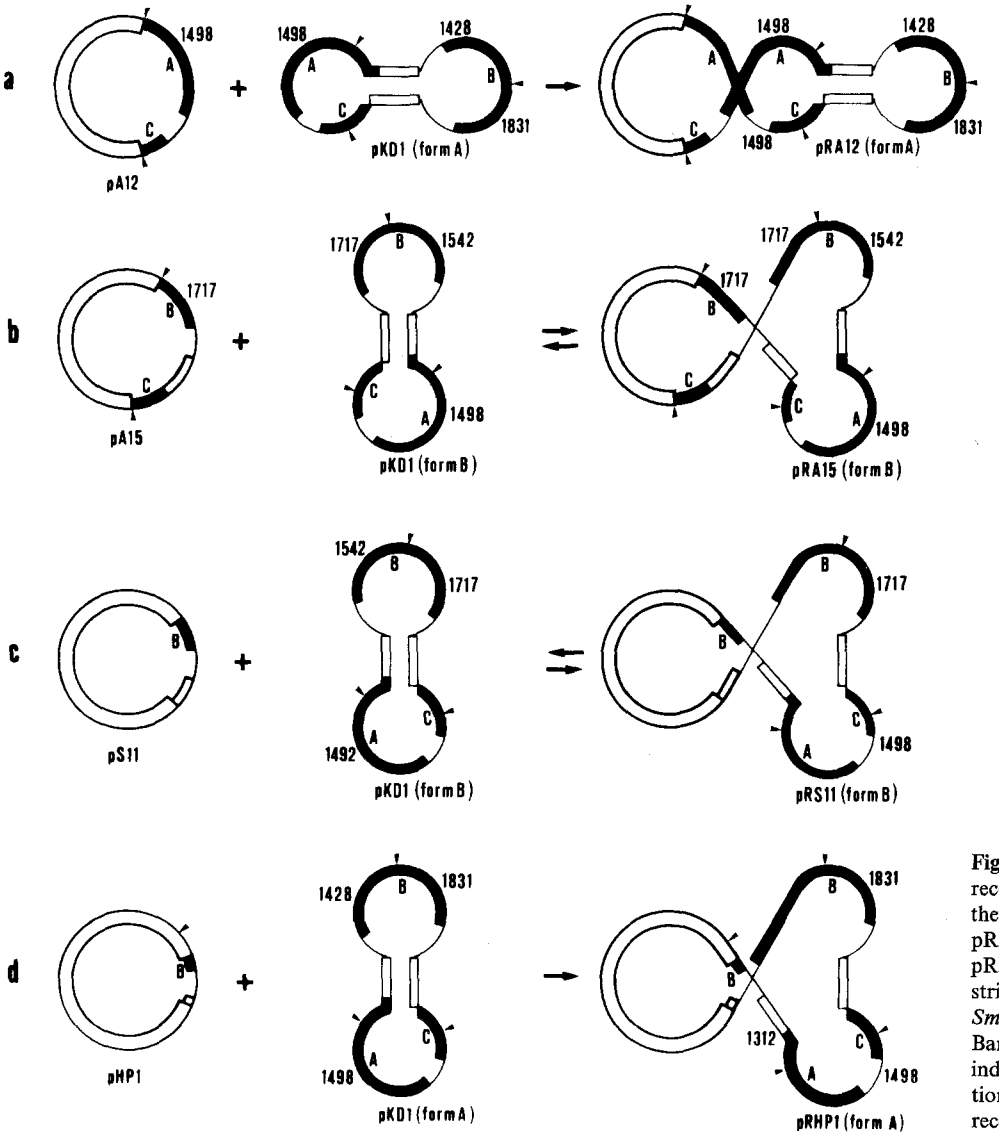

Fig. 4. Schemes illustrating the recombination events leading to the recombinant molecules pRA12, pRA15, pRS11 nd pRHP1, as deduced from the restriction pattern shown in Fig. 3. *Small black arrowheads* indicate BamHI sites. *Single long arrows* indicate homologous recombination, and *double long arrows* FLP recombination. See Discussion

R^+ vectors pS11 and pA15. Figure 3b shows the restriction analysis performed on three *E. coli* transformants obtained from a pA15 yeast transformant. The pA15 plasmid contains an entire IR sequence with an adjacent replication origin. The DNA recovered in *E. coli* clones showed three types of molecules: (1) those identical to the input pA15 (shown in lane 1); (2) pA15 that integrated one molecule of pKD1 (pRA15, lane 2); (3) that pA15 integrated 2 molecules of pKD1 (pRRA15, lane 3). The formation of such molecules is explained by the model shown in Fig. 4b.

The same analysis on a pS11 yeast transformant is shown in Fig. 3c. Again the bacterial transformants contained both the input plasmid and the additive recombinant (pRS11, lane 2). Recombinant molecules containing the other isomeric form or two copies of pKD1 were also observed (data not shown). The recombination event is schematized in Fig. 4c.

The ori^+–IR^- pHP1 plasmid contains the HinPI 375 bp fragment of pKD1 (B form) spanning the replication origin and a small portion (54 bp) of the IR sequence. We were unable to recover any recombinant molecules from DNA extracted from a newly constructed pHP1 transformant and then used to transform *E. coli*. However, after the same yeast transformant was grown for many generations, by restreaking on selective plates, recombinant pRHP1 molecules could be recovered from bacterial transformants (Table 2). These results agree with those obtained from Southern analysis.

In Fig. 3d, lanes 1 and 2, we show the BamHI restriction patterns of pHP1 and its recombinant pRHP1, respectively. The restriction pattern of pRHP1 was exactly that expected for a recombination between the HinPI 375 bp fragment present in pHP1 and the corresponding sequence of pKD1 (Fig. 4d).

Table 2. Characterization of the plasmid molecules recovered from single *K. lactis* transformants after *E. coli* transformation

Input DNA	Number of analyzed *E. coli* clones	Number of *E. coli* clones containing:			
		Input DNA	Recombinant (form A)	Recombinant (form B)	Double recombinant
ori^--IR^-					
pA12a	38	0	19	17	2
pA12b	68	1	35	22	10
pA12c	39	0	15	16	8
ori^--IR^+					
pA10	34	2	14	16	2
ori^+-IR^+					
pS11	85	68	4	8	5
pA15	47	27	15	4	1
ori^+-IR^-					
pHP1	96	96	0	0	0
pHP1[a]	13	10	3	0	0

DNA extracted from a single *pKD1*$^+$ transformant was transformed into *E. coli* and a set of individual bacterial clones were analyzed for the plasmid they contained. The number of analyzed bacterial clones belonging to each set is indicated together with the structure of the recovered plasmids. pA12a, pA12b and pA12c are three independent *K. lactis* transformants

[a]pHP1 transformant after repeated subculturing on selective medium

Table 3. Plasmid stability and copy number in *K. lactis* transformants

Host	Stability (*100 f/s*)		Copy number per *Ura*$^+$ cell	
	pKD1°	pKD1$^+$	pKD1°	pKD1$^+$
Input DNA:				
pHP1	39	44	92.0 (± 44.9)	44.6 (± 13.5)
pHP1[a]	33	70	-	-
pS11	37	60	72.2 (± 26.7)	25.9 (± 5.3)
pA15	29	67	25.3 (± 4.2)	20.5 (± 6.2)
pB11	18	82	16.6 (± 3.1)	19.9 (± 6.3)
pP1	58	61	21.0 (± 6.1)	10.3 (± 2.4)
pE1	97	99	89.4 (± 42.3)	71.3 (± 14.1)
pA10	-	69	-	7.3 (± 1.9)
pA12	-	93	-	27.2 (± 3.6)

For definition of stability, see Materials and methods. Copy numbers include all the plasmids containing the marker sequence pBR322 (input DNAs plus recombinants); the resident pKD1 is not included (see Materials and methods). Values are the average of six (stability) and three (copy number) experiments, with standard deviations indicated in parentheses

[a] pHP1 transformants after repeated subculturing
- Not determined

Stability of yeast transformants and plasmid copy number

In Table 3, we show the stability and the average plasmid copy number of *Ura*$^+$ transformants for various constructs. Stability was measured as the ratio of *Ura*$^+$ cells before and after 7–8 generations of growth in nonselective medium; this allowed us to evaluate the effective loss of the marker during growth on nonselective medium and to avoid including in the data the loss of the marker even on selective medium (for a discussion, see Futcher and Cox 1984).

Stability was very high (from 60 to 99%) for all constructs in the *pKD1*$^+$ context (with the highest value for pE1 and a lower value for pHP1 in newly constructed transformants), while low stability values (18%–

9%) were found in the *pKD1°* context when the constructs were lacking pKD1 genes (pHP1, pS11, pA15) or had a disrupted *B* gene (pB11). For pHP1 transformants, the stabilizing effect of resident pKD1 was apparent only after repeated transfers on selective medium. In this case, we can suppose that the stabilization was a consequence of the selection of molecules recombined with resident pKD1, and not due to the effect in trans of the latter. In fact, the proportion of the pRHP1 plasmids increased with the number of successive transfers (Fig. 2b), while a parallel increase of the stability could be observed (Fig. 2c).

Constructs pB11 and pP1 contained the complete pKD1 sequence except that *B* or *A* gene was interrupted. The transformants of these vectors contained a low copy number, and this level was not increased when resident pKD1 was present. The plasmid pE1, which has all the pKD1 genes apparently intact, showed a high stability and a high copy number in the presence or absence of resident pKD1. However, the presence of all the genes on a plasmid is not sufficient to keep the copy number high since other such molecules (pRA10, pRA12) showed much lower levels. Small constructs (pHP1, pS11) showed a high copy number (inversely proportional to their size) only in *pKD1°* hosts. The general significance of this observation remains to be examined.

For the plasmids lacking the replication origin (pA10 and pA12), the copy number was low, and the stability of transformants was high. In these cases, transformation is a consequence of a recombination with the resident pKD1. In the pA10 transformants, a low percentage of unrecombined vector was present, probably due to the excision of pA10 molecules from the recombinant pRA10 by the FLP activity of the *A* gene. The excised pA10 molecules are gradually lost during cell growth, leading to a slightly lower stability of transformants than with pA12.

Discussion

Four types of pKD1-derived plasmid constructs were examined for their ability to recombine with the resident pKD1 molecules in *K. lactis:* type (A), which bears the replication origin and an IR (pA15 and pS11); type (B), which carries only an IR (pA10); type (C), which bears only the replication origin (pHP1) and type (D), which lacks both the replication origin and IR (pA12). In all cases, we found intermolecular recombinants with resident plasmid. The proportion of the recombinant forms with respect to the input DNA in a given transformant depended upon the type of construct. Similar studies have been carried out in *S. cerevisiae* with 2 μ-derived constructs bearing an IR, with or without the origin of replication (McNeil et al. 1980; Thomas and James 1980). The proportion of these recombined forms among the transformants was 9%–17% and 53%–55%, respectively (McNeil et al. 1980). Corresponding types of pKD1-derived plasmids used in the present study are those of type (A) and (B), and the proportion of their recombinant forms among the transformants was significantly higher (13%–33% and 84% respectively). There have been no reports of recombination with resident 2 μ plasmid when 2 μ-derived constructs lacking the IR sequence are used. In *K. lactis* we have shown that type (C) and (D) plasmids (no IR, with (C) or without (D) replication origin) gave rise to high percentages of recombinants with resident pKD1. This suggests that plasmid recombination plays an important role in the transformation of this organism. Two distinct types of recombination may be considered.

The first is probably mediated by the product of the *A* gene (the supposed FLP recombinase of pKD1) acting on specific sites within the IR sequences, as has been demonstrated in the 2 μ system (Sadowski 1986). Similar recombinase activity has been reported in other yeast circular plasmids (Toh-e et al. 1982; Toh-e et al. 1984; Utatsu et al. 1986). This recombination allows the integration and excision of resident pKD1 molecules with the transforming plasmid when it contains an IR sequence (or the FLP target within IR). When the input DNA lacks the replication origin (as in pA10), the selection will be very much in favour of the recombinant products, because they have all the essential sequences of pKD1 for their maintenance. This explains why the percentage of the recombinant molecules is very high in pA10 transformants. When the input DNA contains the origin of replication, the selection for the recombinants is lower, and other factors, such as the intrinsic instability of each construct, will become apparent.

The second kind of recombination occurs between the resident pKD1 and those input vectors lacking most or all of the IR sequence. This type of recombination is probably mediated by homologous sequences. It is thought to rely on host functions and to take place at a much lower frequency than the other type so that recombinant molecules are only detectable after prolonged growth in selective conditions. This is suggested by the initial appearance of small colonies of the pA12 transformants and by the late appearance of pRHP1 plasmids. In contrast to the FLP recombination, the back reaction is likely to be infrequent in this case, and the input form of transforming DNA will be progressively lost. Indeed, some pHP1 transformants were found to contain only pRHP1 molecules after repeated transfers onto selective medium (Fig. 2b, c). It has been shown (Broach et al. 1982; Bruschi and Howe 1988)

that, in *S. cerevisiae,* the host recombination system can act on the IRs of the 2 μ plasmids to promote intra- and intermolecular recombination in the absence of FLP recombinase or after partial deletion of the FLP target site. Such host functions may also be operating in *K. lactis.*

As noted before, recombination with resident pKD1 plays an important role in the stability of the transformants obtained with some vector constructs. The recombination produces new plasmids that contain all the pKD1 genes in an uninterrupted sequence. The high stability of these recombinant molecules is illustrated in the pA10 and the pA12 transformants: constructs lacking even replication origin become vectors yielding highly stable transformants. IR-based FLP recombination may therefore be exploited for introducing foreign sequences into resident plasmids by means of a vector made of IR and markers only. Furthermore, homologous recombination outside IR sequences seems to produce even more stable transformants than those generated by the FLP recombinase. Intrinsically unstable constructs such as pHP1 can yield stable transformants, due to their conversion to recombinant forms.

Stabilization of a vector by *trans* effects of resident pKD1 was clearly seen in the case of pB11, which has an interrupted *B* gene. No such effects were observed on the vector pP1, which has an interrupted *A* gene. In pP1 and pB11 transformants, copy number was low, even if the interrupted *A* and *B* genes were complemented in *trans* by resident pKD1.

The exceptionally high stability and copy number of the pE1 construct needs commenting on. This plasmid contains the YIp5 sequence at the unique EcoRI site of pKD1, where apparently no genes are present. Such a neutral spot has not been found so far in the 2 μ plasmid. This site might, therefore, be used to introduce any sequences of interest without affecting the stability of pKD1 to any great degree: for example, to introduce a second copy or an over-expressed copy of pKD1 genes in order to study their role in the biology of pKD1.

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