Characterization of a circular plasmid from the yeast *Kluyveromyces waltii*

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(Received 14 June 1991; revised 14 August 1991; accepted 7 October 1991)

A new plasmid was found in the yeast *Kluyveromyces waltii*. This high-copy-number plasmid, named pKW1, is a double-stranded circular DNA plasmid of 5619 bp. It has several features characteristic of the 2 μ -type plasmids: presence of two inverted repeats and four open reading frames, as well as the interconversion of two isomeric forms. However, the nucleotide sequence shows little homology with known yeast plasmids. An *ARS* function was localized within a segment of 545 bp near one of the inverted repeats. Chimeric plasmids carrying this segment efficiently transformed *K. waltii*. A strain of *K. waltii* cured of the plasmid (*cir*^o) was also obtained. In the pKW1 sequence, a functionally neutral region was found at which foreign DNA can be inserted with little effect on plasmid stability. Such constructions carrying the full sequence of pKW1 replicated autonomously in a *cir*^o host and were particularly stable. pKW1-derived full-sequence plasmids also transformed *K. thermotolerans*, but not *K. lactis*.

Introduction

Several circular plasmids resembling the 2 μ plasmid of *Saccharomyces cerevisiae* have been described: pSR1, pSB3 and pSB4 from *Zygosaccharomyces rouxii* (Toh-e *et al.*, 1982, 1984), pSB1 and pSB2 from *Z. bailii* (Toh-e *et al.*, 1984), pSM1 from *Z. fermentatii* (Utatsu *et al.*, 1987) and pKD1 from *Kluyveromyces drosophilarum* (Falcone *et al.*, 1986). These cryptic plasmids, which we may call 2 μ -like plasmids, are found in high-copy-number in the host cell. All have a common structure characterized by the presence of two inverted repeats at which intramolecular recombination occurs to generate two isomeric forms of the plasmid (Hollenberg *et al.*, 1976; Guérineau *et al.*, 1976). Despite the similarity of functional organization, these plasmids share little nucleotide sequence homology. Some homology, however, has been detected at the amino acid sequence level of the plasmid-encoded proteins (Murray *et al.*, 1988).

Each of these circular plasmids has a relatively narrow host range. They can stably replicate only in their natural host and its close relatives. For example, pKD1 of *K.*

drosophilarum can be stably transferred to only a few other species of the genus *Kluyveromyces*, such as *K. lactis* (Bianchi *et al.*, 1987; Chen *et al.*, 1989).

The new plasmid, which we call pKW1 because it was found in a strain of *Kluyveromyces waltii*, has a distinct host range with respect to pKD1. We describe here the molecular and functional organization of pKW1. Various derivatives of pKW1 can be used as efficient vectors for gene manipulation in *K. waltii*.

Methods

Strains and media. pKW1 was isolated from *Kluyveromyces waltii* Kodama, CBS 6430 (type strain). The *K. lactis* (*Kluyveromyces marxianus* var. *lactis* Johannsen et van der Walt) strains were MW98-8C (α *uraA argA lysA*, K⁺, pKD1⁺) and its isonuclear clone MD2/1 (pKD1⁺). All the type strains of *Kluyveromyces* species were obtained from the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. The *Saccharomyces cerevisiae* strain was S150-2B (*Mat a ura3 leu2 trp1 his3 cir*⁺). Complete medium (YPG) for yeast growth contained 1% (w/v) Bacto yeast extract (Difco), 1% (w/v) Bactopeptone (Difco) and 2% (w/v) glucose. Minimal medium W contained 0.67% (w/v) Yeast Nitrogen Base without amino acids (Difco) and 2% (w/v) glucose. For plating, these media were supplemented with 2.5% (w/v) Bactoagar (Difco). The *Escherichia coli* strains used for recombinant plasmid construction were JM83 (Vieira & Messing, 1982) and MC1066 (Casadaban, F⁻ *lacX74 hsdR rspL galU galK trpC9830 leuB6 pyrF*::Tn5). Complete medium for *E. coli* was LB (Maniatis *et al.*, 1982) supplemented with either ampicillin (40 μ g ml⁻¹) or kanamycin (100 μ g ml⁻¹), with or without agar (1.5%, w/v).

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Abbreviation: IR, inverted repeat.

The nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession number X56553.

Preparation of yeast plasmids. Small-scale crude preparations of yeast DNA (2–5 ml cultures) were obtained according to the procedure of either Cameron *et al.* (1977) or Sherman *et al.* (1983). For large-scale purification of pKW1, cells from 21 early stationary-phase cultures were harvested by centrifugation ($13\text{--}15\text{ g cells l}^{-1}$), washed with $150\text{ ml } 1\text{ M-sorbitol}/1\text{ mM-EDTA}$, and resuspended in $100\text{ ml of } 1\text{ M-sorbitol}$ containing $30\text{ mg Zymolyase 20T}$ (Kirin Breweries Co., Tokyo, Japan). After incubation for $1\text{ h at } 30^\circ\text{C}$, the protoplast suspension was mixed with $5\text{ ml } 10\%$ (w/v) SDS and $5\text{ ml } 0.5\text{ M-EDTA}$, pH 7.0. After vigorous swirling, the lysate was incubated at 50°C for $1\text{--}2\text{ h}$. Potassium acetate was added to a final concentration of 1 M , and the solution was cooled on ice for 2 h . Precipitates were removed by centrifugation (Sorvall rotor SS34, at $15000\text{ r.p.m. for } 30\text{ min}$). From the supernatant, nucleic acids were precipitated at 4°C by the addition of $2\text{ vols } 95\%$ (v/v) ethanol. The precipitates were collected by centrifugation, washed with 70% (v/v) ethanol and dried under vacuum. The pellets were dissolved in $40\text{ ml } 5\times\text{TE}$ (TE is 10 mM-Tris/HCl , 1 mM-EDTA , pH 8.0). After the addition of 40 g CsCl and $4\text{ ml ethidium bromide}$ (10 mg ml^{-1} stock), the solution was centrifuged at $60000\text{ r.p.m. for } 6\text{ h}$ (Beckman rotor 65 Ti). The band of supercoiled DNA beneath the chromosomal DNA was collected, and purified by a second cycle of isopycnic centrifugation. Ethidium bromide was removed by isopropanol extraction, and the plasmid DNA was dialysed against $0.1\times\text{TE}$.

Other plasmids. The vectors used to clone pKW1 were YIp5 (Botstein *et al.*, 1979) and pKan21. The latter was constructed by inserting the kanamycin resistance gene from Tn903 (a $1.3\text{ kb } AclI$ fragment from the Kan^R gene block, Pharmacia) into the *NarI* site of pUC19. The pKD1-derived plasmid, pCXJ-kan1, contains the total pKD1 sequence (Chen *et al.*, 1986), the *URA3* gene of *S. cerevisiae*, the Kan^R gene and the pUC19 sequence. The yeast 2μ plasmid was isolated from *S. cerevisiae* S150-2B, pKD1 from *K. lactis* MD2/1 (Bianchi *et al.*, 1987), pSR1 from *Z. rouxii* CBS732, pSB1 and pSB2 from *Z. bailii* ATCC 8766, and pSB3 and pSB4 from *Z. rouxii* CBS 4357.

Yeast transformation. *S. cerevisiae* was transformed by the procedure described by Beggs (1978), and *K. lactis* by the method of Bianchi *et al.* (1987). The *URA3* gene of *S. cerevisiae* was used to complement the *uraA* mutation of *K. lactis* hosts. Transformation of *K. waltii* and selection of G418 resistant clones were carried out by a protoplast procedure essentially similar to that described for *K. lactis* transformation by Chen & Fukuhara (1988). The level of G418 was $200\text{ }\mu\text{g ml}^{-1}$.

Transformation of *E. coli* and manipulation of DNA. Published procedures were followed (e.g. Maniatis *et al.*, 1982). For nucleotide sequencing, pKW1 DNA was subcloned into overlapping fragments in the cloning/sequencing vector pTZ18R (Pharmacia) and the dideoxy chain termination method of Sanger *et al.* (1977) was used.

Results and Discussion

Detection of pKW1

Crude DNA was prepared from a small-scale culture of *K. waltii* CBS 6430 and analysed by agarose gel electrophoresis. In addition to the chromosomal DNA, a fast-migrating DNA was detected by ethidium bromide staining. The DNA band migrated more slowly than the reference pKD1 DNA, 4.7 kb , of *K. drosophilae*. Comparison of the intensity of staining suggested that the amount per cell of this DNA was roughly comparable

Fig. 1. Restriction digestion patterns of pKW1. The plasmid purified from yeast was digested by *Bam*HI (lane 1) and by *Pvu*II (lane 2). In each case, one sees two pairs of fragments with a constant sum (5.6 kb) of molecular masses ($4.8 + 0.8 = 5.6$; $2.9 + 2.7 = 5.6$, etc.). The molecular mass reference (lane 3) is a mixture of λ *Hind*III and ϕ x174 *Hae*III digests.

to pKD1 and other known circular plasmids, that is, in the order of 60 copies. The new DNA was named pKW1. Other strains of *K. waltii* (seven independent isolates, generously provided by Dr M. A. Lachance, University of West Ontario, Canada) were devoid of pKW1 or related plasmids.

Purified pKW1 DNA was analysed by digestion with various restriction enzymes. Digestion with *Eco*RI, *Sph*I, *Sal*I, *Cla*I, *Nhe*I and *Bgl*II gave rise to a single fragment of 5.6 kb . *Pst*I or *Xba*I digests produced two fragments which again gave a total molecular mass estimate of 5.6 kb . *Bam*HI or *Pvu*II digests gave four fragments whose sum was 11.0 kb ; in both cases, the four fragments could be interpreted as two pairs of 5.6 kb each (Fig. 1).

Fig. 2. Restriction maps of the A and B forms of pKW1. The two forms of pKW1 differ in the orientation of the right half of the molecule. A, B, C and D are open reading frames, IR1 and IR2 are inverted repeats.

These results suggested the presence of two isomeric forms of a circular DNA as in the case of other 2 μ -like plasmids.

Cloning in *E. coli*

The existence of two isomeric forms was confirmed by cloning pKW1 DNA into *E. coli*. pKW1 was opened at the unique *Cla*I site and cloned into the *Acc*I site of the pUC19-derived vector pKan21. Hybrid plasmids were recovered in four different combinations: the A and B forms of pKW1 inserted in the two possible orientations into the bacterial vector. Using these clones, the restriction maps of the A and B forms were established,

as shown in Fig. 2. pKW1 is composed of a pair of inverted repeat sequences of about 300 bp each and two unique sequences of 2.6 and 2.3 kb. These maps confirmed that internal recombination occurs at the inverted repeats to generate the two alternative forms in the yeast host.

Homology of nucleotide sequence with other yeast plasmids

To see whether pKW1 has any identifiable relatives among the known 2 μ -like plasmids, DNA sequence homology was examined by Southern hybridization under moderately stringent conditions (0.6 M-Na⁺, 65 °C), using ³²P-labelled pKW1 as probe. No detectable hybrids were formed with 2 μ DNA of *S. cerevisiae*, pSR1, pSB3 and pSB4 of *Z. rouxii*, pSB1 and pSB2 of *Z. bailii*, or pKD1 of *K. drosophilum*.

Nucleotide sequence of pKW1

Fig. 3 shows the nucleotide sequence of pKW1. The total length is 5619 bp. The two inverted repeats, 316 bp long, separate two unique sequences of 2643 and 2344 bp. Four open reading frames are present: A (433 codons), B (241 codons), C (306 codons) and D (289 codons). The inverted repeat sequence has a short stretch of sequence homologous with pSM1 inverted repeats as shown in Fig. 4. Although the open reading frames show little sequence similarity with other circular plasmid genes, the A gene product has unambiguous amino acid sequence homology with the FLP recombinase gene of the 2 μ plasmid and its equivalent in other plasmids (Fig. 5), suggesting that the A gene may be involved in the interconversion of the isomeric forms of pKW1. While pKD1 of *K. drosophilum* has three open reading frames (Chen *et al.*, 1986), pKW1 has a fourth gene which might have a function equivalent to that of the D gene of the 2 μ plasmid.

Localization of ARS function

In order to identify the replication origin in the pKW1 sequence, we initially used a *ura3* mutant of *S. cerevisiae* as a transformation host, since no genetic markers are available in *K. waltii*. Different segments of pKW1 were cloned into the yeast integrative plasmid YIp5 (see Fig. 6). Each chimeric plasmid was used to transform *S. cerevisiae* and *K. lactis* Ura⁻ hosts. Some of the pKW1 fragments were also cloned into pKan21 (see Methods) and used for the transformation of *K. waltii*.

The transformants in *S. cerevisiae* were detected by the Ura⁺ phenotype due to the complementation of *ura3*

ATCGATTGCC AGCAACAGGG TTTTGC GCGT ATGTCTCTTG TGGATACTAT GTAAACAAAA 60
ClaI *B* gene *IR1*
 CAATCAATGT ACAAAGAGCA CAGCGGCCGG CAGGTGGGAG GACCCCTCTGA TGAGCCGGGG 120
 ATATGGCGCT CCCGCGCGTC TAATATCCGG ATTGGACTGG AGGAGGACCA AGGTTTCCTC 180
XhoI
 GAGGTCATGT GCCGCATCAT GAGGGACGAA AAATGGTAAG GAATAGACCA TTCCTTACCA 240
 TTTTTCGTCG CCCGTGAGAT TTTCCATTT CCCGTTCCCTT ACCATTTTTTC GTTCCGCACG 300
 ACCCTATCAC ATTGTATATT GAAATCTACT TCCCTTCATA CCGCTTGGCC ATCGCGTAGG 360
 AATGAACATA TGAGCCCCCT CTAGTTCCCTC ATGTTGTTCT CAATCGACCC TTCCATCGTG 420
IR1 *C* gene
 AAGGGATCTC CTCTTGAGGC AATGTCGACG TTCGTACCCT CTTCTACGG GTCACGGGTT 480
SalI
 CGTCTGTAAC ACTTCTTCTT GGTCTCCCTC GGCCTCTTTT ACTTGGCTGT GGCCTGGTAT 540
 TTTTGAGTTC ATCGAATAGG CGCGGGTTTT CTTTTACTTG AGGCCAGTCT TGAATCTCTG 600
 AACCGAGAGA GGTAGAGAA GTTATTCTTT TTAGATCATT TAACATGTCC TTGATGTCTT 660
 CTGGCGTTGT TTCGAAGCAC ATCAGTCCCC TTGAGCGATA TCTGTCGTAA TTTGCCAGTA 720
 TTACGTTGTG CGTTCTTCC ATGTTTTTCAG GGGCCACATG CTTTAATGCC TGCATAAAAA 780
 CACTTCTGAC AAGTGACCAA TCTGCGAATG CAGGCTCGTT CGCCAATATC AATCTCTGTA 840
 GAGGAACACC ACGATAGCTT GTTGTGCCCT TGCAGATAGT GTTCAAGAAT AGACTGAGGT 900
 AGCCACGTAC GTCGAGCTTT ACAGACCTGG TGCCAACGCC AGAAAGAAGG GCGAAGGGGT 960
 CCTCTTCAAT CGGAAAACGG GTCAGGCTAT CCACACGGTA CTCTCTATAT GTACTACGAG 1020
 TCATCGTTCG GTACTTTGCG AAGTGCAGGC CGTGCGGATT ATAGTCAATC TGGAAAGAAT 1080
 CCTGCTCATG TGGCTCTTTC TCCCCATTAT CCTCTTCAGA GTTCAGCGAG CCAGATGGCA 1140
 GCTCGGCTGG CTCTCGCAGG TACGTGGGAT CGGGCTCACC TCGATCCACC GTCATCTCAT 1200
 CAAGATTGCA CATAATCTGC ATCAGTCTCA ACGTCATATC GACCGCGGAA AAGGCGTCCG 1260
 AGTAGGAAAC ATGCAGTTTG GAAAGGTCTA ACAGCTTGAG GCAGTGCATC TTGGCCTCTC 1320
C gene
 TCTCTGTCAG TCTTTCTCTG TTTTGACCAA TTTTCTCAA GTGTATTACA TTTGTTTCTT 1380
 GGATTGCAAA AATGTTGCGA GTACTGCGTG TTCTCGTTCA AAAACAGCGT TCGCTGAAAA 1440
 TTTAGGAGTT CAGATGCAAC GCGTAGTGCA AATGGAGGAT TCAAGTTGCA GTAACAATAA 1500
 CATGGAACAC CAAGGATCAG TGTTTCGAGGA GCTTATCTCC AAAAATCTTA TGAGCCTGAT 1560
 GGAAGAATTG ATGTCTATGC TCACTAATGA GAAGGAGTTC CAACGTGAAA GGTTGCGGTC 1620
 TCTCCTAGCC TACATGATAA TGGCTACTGG TGAATTGGAA GAGAAAAAGC TCAGTACATT 1680
 TACCAAGTAT TCCCGACGGA TCAGGCAGAC GGTAGAGTTC GACAGCAACA ACCAAATCGT 1740
 AAGATTGAG TACCATTGTA AGAATCCCAC AGAGCTCAAG GAGACGCTGG ACAAGGCCTT 1800
SacI
 TAAACCTGTC GTGTTTGAAA TCAAGTCCAA AAAGAAGGTT GTCTCCATGC TGGAGCTAGC 1860
XhoI
 TGCGAAGCTC GACAAAAGGG GATCAGATTC AGCGGTGGT ACGGTAGCTA GTGAGGTCTC 1920
 GAAGCTTGTC CGGGAGGAAG AAATTTGGCT TCTTCTCGTG AACGTGAAGA ATACTATCCA 1980
 GGAAAAGGTG CGCAAATCAT CGCTAAGAGC GGAATTGACG TATATTTTGA CAGCCTCATT 2040
 CTTCAATTGT TGCAGACATA GCGATCTCAG GAACGCAGAC CCCGCAACAT TTGAGCTGGT 2100
 GCCAAATAAG TATGTGGGCC ACGTTGTCCG GGTTTTGGTG TCGGAGACCA AGACCCGAAA 2160
 GCCGCGGTTT ATATACTTTT TCCCTGTCAA TACGCCCGCG GATCCTCTAG TAGCGCTTCA 2220
BamHI
 TGATTTGTTT TCGAGCACGT TTCCTTCCAA AAAGAGTCGG ACGTCCGAAA GAAAGCAGGA 2280
XhoI
 ACAGGAATGG CAGATCGTTC GCGACGCATC AATCAACAAC TATGACCGGT TTGTTGGTAA 2340
 GCACGCTACG GAATCTGTCT TTGCCATCTT GCATGGTCCC AAATCACACT TGGGCCGGCA 2400
 CTTGATGAGT TCCTACTTGG CGTATACCCA CCATGGGGAA TGGGTCTCAC CATATGGGAA 2460
 TTGGTCAGCT GGGAAAGGAA CCATTGAAAG CAGCGTGGCA AGGGCCAAGT ACGCACATGT 2520
 TCAAGCCGAG ATCCCAAGCG ATCTTTTCCG CTTTCTGTCT CAGTACTATC AGGAATCAAA 2580
 ATCGGGCGAT TTCGAGCTTA ACGACACCAG CAAAGACCCA ACAAAGCTGG TACGGCACTC 2640
 GGCTAGTCAA CTGGAATCA ATCGAACCTA TGGTCCATGG AGTAGATTGG TTAACAAGGA 2700
 TGTTTTAGGC TTTGTTTCATT CCTACGCGAT GGCCAAGCGG TATGAAGGGA AGTAGATTTT 2760
IR2
 AATATACAAT GTGATAGGCT CGTGCGGAAC GAAAAATGGT AAGGAACGGG AAATGGGAAA 2820

ATCTCACGGG	CGACGAAAAA	TGGTAAGGAA	TGGTCTATTC	CTTACCATTT	TTCGTCCCTC	2880
ATGATGCGGC	ACATGACCTC	GAGGAAACCT	TGGTCCTCCT	CCAGTCCAAT	CCGGATATTA	2940
GACGCGCGGG	AGCGCCATAT	CCCCGGCTCA	TCAGAGGGTC	CTCCACCTG	CCGGCCGCTG	3000
TGCTCTTTGT	ACATTGATTG	TTTTGTTTTA	GTATTACCTG	ACAATCATTT	TATATTTTGT	3060
CAGTATTTCT	TGATATCTGG	CCCAAATGAA	AGTACAAATA	CAAGTACAAG	TACAACACCT	3120
ATTCATTGTT	ACCGTATCTC	TATACTATTA	TCCTTATTTT	CTGCCTAATT	AACTACTTTC	3180
TACCGGTGCG	TTCTTAAAGC	TGAGTAGGCA	CTTCAGCCCG	AAGTGAATAT	CGCTGGCCTC	3240
ATTATCTGTT	TGTTGTATGG	CCAATGAAAC	TCCAACCGAT	TTGATCCACT	TGCAAGGACC	3300
GTAGGATAGA	CTTATTTTAA	CCATCCCAT	ATCTGTGCAG	TGGACACCTC	TGTTTATCTT	3360
TACCCGCCCT	TCAGAGAACA	TAATCTGTCT	TACAAAAGAG	TAGACCTCAT	TTTTGGCGAG	3420
AAGCCTCTTC	TGTTTGACCT	CATCTAGAAG	CTTTTGGGCT	CTCCAGCGC	AATCAGATAG	3480
TCTTGTGAGA	GTGTCCTCTT	CGTCACCATG	CTCATCAGAA	GGAGCTGGTT	GCGTTCCAAT	3540
TGAGAAAGCT	CGTCCCAAAA	GGCTGCATGG	TCTATAGGTG	GTCCCGAACC	CGAGGATTCA	3600
CCTTGAGACT	CATTGTCCAC	AGTAGGCTCC	TGATCCTCTG	CAGCAGCCGC	TTGCTGAATG	3660
GATTCTGCTC	TTGACATTAC	CATTTTCGAGA	AGCCATAGTC	TTCGGATGGT	TCTAGCAGAT	3720
CTTTCAACAT	CCAAACTGGA	ACCGAACCAT	TGCCGACAAC	TTATTCTAAC	CTCAAGCTTT	3780
GAGAGGTTCT	CATACTTGGA	TTGTGCAGCC	TCAAGATCCG	TAAGTTCGGT	GAAGAAATCT	3840
AAAGCTTCGC	GTTTTGGACC	TAACCGAATG	ATGATTGGGT	GCCGTTTTTCG	TGGATCCAGA	3900
AATGGTGTA	GCTCCTCTAT	ACTATCATTC	GTGGGACACT	CTATCCCTTC	CCCAATATGC	3960
TTCTTTACAA	TAGAAGGTAG	CTGCGTATAC	TTGTTCCGAA	CAACAAAGAT	CTGACTCTCT	4020
AGCCGCTTTA	CTATTGTGTT	CAACACAACG	TAGGGTTTTG	GCCACTCAAA	TGTTAATTCT	4080
GATCGAGCTG	CTGATGCTGT	GTTCTTGTTG	ATCATGTATG	AATAGTAGAA	CAATGCCAAT	4140
TGAGGATGCT	CACTGTATTC	CTTAGGCAGT	TTGGTGCTGC	CATAAGGTGA	CACCAGCTCC	4200
TTTAGCTGTG	GAACATAGAG	TACATCAACA	GTTCTACTA	GACAACATTC	CTTGAGACAA	4260
TTATGATAGC	CATCATGGTC	GGTTATCTTA	GGTATTTTTT	GAGCTTCACT	CATCTTCGAA	4320
GCAACCGTGA	TAGATTTCGAT	TGAAGTTCAA	ATTCTTATCT	AGATGGTGTA	TTTGTTTTATC	4380
ATAATTTACA	ATACAGTCTG	TTTTAATTTG	CTCGAAGTTG	CAGTGAAAGA	TGTAAAAAGG	4440
GGCTTCATTT	TGCACTACAG	ACTTACCCTG	ATGTAAAAAA	TTTTTCATCAT	AAAAGCAATT	4500
TCTCGTATAC	AAAATGCAGT	TTGTTTCGTA	TACAGCAAAAG	CATAGAATAC	ACTGTACACT	4560
GCATTTTAC	TACAAGAAAA	GTTTTTTTTG	CTGCTGTGAC	TGGAATGCTG	CAGCAGTACC	4620
TAGATAGAAA	AATGGCATAA	AACTGAAATT	TTATAGTCAT	TTTTTCGTGTC	TTTCATTCAA	4680
TTTTTCTCG	CAAAAGTTTT	CTACAAAAGC	AGTCAAAAATT	GCAATAAGTA	TACACTAATT	4740
ACAAGGCGTC	TGAGCGCGTG	ACTTGAGCGC	GTGACGTAAT	CGCGAGCTAC	GAAAGTTGTT	4800
TGGGCCTCAG	ACATCGGATC	GACAGAAGAG	GTAAGAATAT	TGGGAAAGTA	CATTCAATTA	4860
CCACAACAAT	CGAGAGATTA	GTGGAATTCA	GTCATTAATG	AAAGGTAGGG	TAGTCCGCCT	4920
ACTCTTAGTT	CTACATTCAG	AATACGCATG	CAGTCCTCAA	GTTCTGATGA	AGACGACCTA	4980
ATTGACCCTA	TAATTCATCC	CAAATCGTTC	TATAGGGCGG	CTAACGAGAT	ACCGAGAGAC	5040
TTTTTGGTTG	CGATCCCCAT	CAGCGCCTAT	GTTTTTAGCG	TATTTGCTAA	ATCAGTACGA	5100
GATGACTTAC	AGGGGCATTT	AACGGCGCGA	GATATGGCAT	TAGCTTATCG	TGAACGGCAG	5160
TACTTTCACA	GACGCTGGGA	GACACGAAAC	GACCAGCTTG	AGATCCCAGA	CTGGTCTGAG	5220
ATCCAGAAAT	GGTCTCTCGG	GTTACTGGAT	CGCCCTCCTT	GTATCACTGT	GGATCTAGCA	5280
AGAGAACTGC	GCGAACTATC	TCAAAAATGG	ATCGGAGCAT	TCGATCTGGG	ATCGAAGATG	5340
TCTGGCAGGC	TTCTTCTACA	GCTTCTGTAC	ACCCAGCTGT	CATGCCCAAA	TGAGGCTGTT	5400
TTCAATAAGC	TTTACTGCCT	CGTCAAACCTA	CTAAACAAGG	ACGTAAATCG	TGCGGACCGA	5460
GCCCTTATGG	ACTCTGTATT	GAGACCACTT	TTGTGCGAGA	ATCCATACAT	GGGTGAACCTA	5520
GATGAAGAAA	TACTTGATAA	GATATGGTCC	AATTTGACTG	AAATGAGAAG	TCAAGAGTGG	5580
AAACGTATAG	CGGAAGCGTT	GTCAGGCGAG	AATAATGAC			5619

Fig. 3. Nucleotide sequence of pK W1. The sequence of form B is given. The numbering starts at the first base of the *Cla*I recognition sequence. The major restriction cleavage points used in vector construction are as follows: *Cla*I (nucleotide 2), *Xho*I (178, 2230, 2898), *Sal*I (444), *Sac*I (1776), *Nhe*I (1855), *Bam*HI (2200, 3892), *Xba*I (3443, 4358), *Bgl*II (3717), *Eco*RI (4884). Inverted repeats (IRs, 316 bp) are at positions 53–368 and 2713–3028. The putative target sites of the FLP recombinase are shown by horizontal arrows in IRs. Four open reading frames are present: A, 1454–2752 (1299 bp); B, 4948–51 (723 bp); C, 392–1309 (918 bp, on opposite strand); D, 3447–4313 (867 bp, on opposite strand).

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      . . . . .
pSM1  AGATGAGAGAAATGGAAAGGAAT GAACC ATTCCTTTCCATTTTCGCTCGCTT
      **      ** ***** *****      *** ***** ***** *
      . . . . .
pKW1  AGGGACGAAAAATGGTAAGGAAT AGACC ATTCCTTACCATTTTTCGTCGCC
      *      ** * **      *** * * ***      ** * **
      . . . . .
pKD1  ATATCAGACATTTGTCTGATAAT GAAGC ATTATCAGACAAATGTCTGATAT

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Fig. 4. Comparison of the putative recombination sites of pKW1, pKD1 and pSM1 within the IR sequences. The IRs contain a palindromic sequence which is the putative recombination site. The palindromes are marked by the dots over the sequence. Identity of bases is marked by ★.

Fig. 5. Homology of the deduced amino acid sequences between pKW1-A, 2μ-FLP and pKD1-A gene products. Amino acid sequences were compared by dot-plot analysis at four matches per 10 residues. Top; pKW1-A gene vs 2μ-FLP gene. Bottom; pKW1-A gene vs pKD1-A gene.

mutation (Table 1). The recombinant plasmids containing a specific region near the end of one of the inverted repeats were capable of transforming *S. cerevisiae* at high frequency. This region was narrowed down to the 545 bp *Xba*I–*Xho*I segment (nucleotides 2898–3443). An 11 bp sequence (5′ TTTTATATTTT 3′), similar to the *S. cerevisiae* ARS consensus (5′ A/TTTATA/GTTTA/T 3′) was found within this region (nucleotides 3048–3059). Note in Table 1 that the recombinant plasmids pNEA2 and pNEB1 which gave no transformants did not contain this segment. That this segment functions as an

Table 1. Transformation of *S. cerevisiae* by pKW1-derived plasmids

Different DNA fragments of pKW1 (see Fig. 6) were cloned in YIp5 and used to transform *S. cerevisiae* S150-2B (*ura3⁺*). The structures of the recombinant plasmids are shown in Fig. 6. pSK1 contains the replication origins of both 2μ and pKD1 (Chen, 1987). The stability is expressed as the percentage of *Ura*⁺ cells after 10 generations of growth in liquid YPG (non-selective conditions). At least three separate transformants of each plasmid were tested. The values shown are the mean of these experiments. Data from repeated experiments generally varied less than 10%.

Recombinant plasmid	Presence of <i>Xba</i> I– <i>Xho</i> I 545 bp segment	<i>Ura</i> ⁺ transformants per μg DNA	Stability (%)
pKWS1	+	4400	0.58
pBNA1	+	1200	0.14
pNEA2	–	0	–
pBNB1/A3	+	7600	0.27
pNEB1	–	0	–
pXXY2	+	4000	0.12
pSK1	+	4500	60.00

ARS element in the natural host *K. waltii* was confirmed by transformation of *K. waltii* (Table 2). The transformants were detected by the G418-resistant phenotype due to the Kan^R gene carried by the transforming DNAs. The construct pXXK3, which contained the 545 bp *Xba*I–*Xho*I segment, showed a high frequency of transformation in *K. waltii*.

In other 2μ-like plasmids, a ‘stability locus’, acting in *cis* with the ARS region, is believed to play a role in the plasmid partition during cell division (Kikuchi, 1983; Jearnpipatkul *et al.*, 1987*b*). Such a locus still remains to be defined in pKW1.

Curing of pKW1 from *K. waltii*

The original host strain, CBS 6430, which harbours pKW1 was transformed by the plasmid pKWS14 (see below). This plasmid, disrupted within the *C* gene at the unique *Sal*I site, is dependent on the presence of the resident pKW1 for its stable maintenance. G418-resistant transformants were grown for 75 generations in

Fig. 6. Structures of recombinant molecules carrying from pKW1. (a) From the A form of pKW1, various restriction fragments were isolated as indicated by filled, open and dotted segments. They were cloned into either YIp5 or pKan21. The resulting recombinant molecules were designated pBNA1, pNEA2, pXXY2 and pXXK3, respectively, as indicated. (b) From the B form of pKW1, two restriction fragments indicated by filled and open segments were cloned into YIp5 to obtain pBNB1/A3 and pNEB1, respectively. (c) Structure of the plasmid pKWC11 in which the total pKW1 sequence in A form was joined at the *Cla*I site to pKan21. (d) Structure of the plasmid pKWS1 in which the total pKW1 sequence in A form is joined at the *Sal*I site to YIp5.

Fig. 7. Stability of pKWC11 and pXXK3 in the *K. waltii* *cir*^o strain. pKWC11 has the totality of the pKW1 plasmid sequence, while pXXK3 contains only the replication origin region of pKW1. The transformed host was KW18. The stability is the percentage of cells retaining the transformed phenotype (resistance to G418) after the indicated number of cell divisions under non-selective conditions (liquid YPG medium).

YPG containing G418 (1 mg ml⁻¹). The cells were transferred to YPG (without G418) and grown for a further 10 generations. Cells were finally plated on YPG agar plates. Since pKWS14 is unstable in non-selective medium, many drug-sensitive clones were isolated, as revealed by replica plating on G418-containing plates. Among 20 such clones, five were found to be devoid of both the input vector and the resident plasmid, as verified by electrophoresis and dot-blotting hybridization of cellular DNA. One of these clones (strain KW18) was retained as a *cir*^o host and used in the transformation experiments described below.

Full-sequence derivatives of pKW1 and their stability

pKWC11, which contained the total pKW1 sequence, also efficiently transformed *K. waltii*. In this case the transformants showed particularly high stability (Fig 7). This high stability was not due to a possible integration of the resistance gene into the chromosomes, but due to the presence of the replicating plasmid (data not shown). pKWC11 had been constructed by inserting the pKan21 sequence at the unique *Cla*I site of pKW1. Therefore, the *Cla*I site seems to be a functionally neutral point at which insertion of a foreign sequence does not significantly affect the plasmid stability. The presence of such neutral points has previously been reported in other 2 μ -type plasmids: the *Eco*RI site of pKD1 (Bianchi *et al.*, 1987; Chen *et al.*, 1989), the *Sna*BI or *Xma*III site of the 2 μ plasmid (Chinery & Hinchliffe, 1989; Bijvoet *et al.*, 1990) and at the end of the *R* gene of pSR1 (Jearnpipat-

Table 2. Transformation of *K. waltii* and *K. lactis* by pKW1-derived recombinant plasmids

pKWC11, pKWB11 and pKWS14 contain the entire pKW1 sequence and Kan^R marker. pXXK3 has the *ARS* segment of pKW1 as well as Kan^R (see Fig. 6 for their construction). pCXJ-kan1 contains the entire pKD1 sequence (see Methods). The stability of transformants as defined in Table 1 is given in parentheses.

Recombinant plasmid	Replication origin	G418 ^R transformants per µg DNA			
		<i>K. waltii</i>		<i>K. lactis</i>	
		(<i>cir</i> ⁺)	(<i>cir</i> ^o)	(<i>cir</i> ⁺)	(<i>cir</i> ^o)
pKWC11	Total pKW1	36000 (98%)	8000 (100%)	0	0
pKWB11	Total pKW1	21000 (98%)	10000 (4.6%)	0	0
pKWS14	Total pKW1	10000 (92%)	8000 (29%)	0	0
pXXK3	<i>Xba</i> I- <i>Xho</i> I 545 bp from pKW1	35000 (49%)	10000 (2.8%)	0	0
pCXJ-kan1	Total pKD1	12000 (94%)	13000 (69%)	10000 (62%)	9700 (100%)

Table 3. Host range of pKWC11

The species are those described by van der Walt & Johannsen (1984). % GC is the reported guanine-cytosine content of chromosomal DNA. Stability is defined in the legend of Table 1.

Species	Strain	% GC	Transformation frequency	Stability (%)
<i>K. waltii</i>	CBS 6430	45.6	2400	100
<i>K. thermotolerans</i>	CBS 6340	46.2	4000	25
All other species of <i>Kluyveromyces</i> *		33.1-42.9	0	-

* The species tested were: *K. blattae* CBS 6284; *K. phaffi* CBS 4417; *K. polysporus* CBS 2163; *K. lodderae* CBS 2757; *K. africanus* CBS 2517; *K. aestuarii* CBS 4438; *K. delphensis* CBS 2170; *K. marxianus* varieties *vanudenii* CBS 4372, *lactis* MW98-8C, *drosophilae* CBS 2105, *wikenii* CBS 5671, *bulgaricus* CBS 2762, *marxianus* CBS 712, *dobzhanskii*, CBS 2104; and *K. wickerhamii* CBS 2745.

kul *et al.*, 1987a; Ogawa *et al.*, 1990). These sites are in an intergenic region of the plasmid. The *Cla*I site of pKW1 is, however, within the *B* gene, very close to C-terminus (see Fig. 2). Insertion at this site does not appear to affect the function of this gene. In contrast, two other plasmids, pKWB11 and pKWS14, obtained by insertion of pKan21 in the *Bgl*II site (within the *D* gene) and the *Sal*I site (within the *C* gene), respectively, were highly unstable in *cir*^o hosts (Table 2). The recombinant plasmids of the pKWC11 type are therefore the vectors of choice for the propagation and expression of foreign genes in *K. waltii*. Since the replication of pKWC11 does not require a *cir*⁺ host, and since it contains the Kan^R marker, it can be directly used for the test of host range among various yeast species (they are in most cases sensitive to G418), as shown below.

Host range of pKW1

Although *S. cerevisiae* can be transformed by pKW1-derived plasmids, the transformants are quite unstable as shown in Table 1. None of the pKW1-derived plasmids were able to transform *K. lactis* (Table 2). In contrast, the pKD1-derived 'full-sequence' vector pCXJ-kan1 gave stable transformants at high frequency in both *K. lactis* and *K. waltii*, confirming our previous observation (Chen *et al.*, 1989).

In order to see the host range of pKW1, all the species of the genus *Kluyveromyces*, according to van der Walt & Johannsen (1984), were tested for transformability by pKWC11. Table 3 summarizes the results. Only *K. thermotolerans* gave rise to transformants with a frequency comparable to that of *K. waltii*. This result may

be correlated with the fact that *K. thermotolerans* and *K. waltii* make a distinct group within the genus, as characterized by the high GC contents of their chromosomal DNA. The stability of *K. thermotolerans* transformants was variable, very high in some clones. The absence of transformation with other species tested so far does not necessarily indicate an inability of the vector to transform them. Transformation under varied conditions remains to be examined for each species.

This work was supported in part by the Centre National de la Recherche Scientifique (AIP 6931), the High Technology Development Program of China, Rhone-Poulenc Santé and the Commission of the European Communities (BAP-0026F).

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