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The host range of the pKD1-derived plasmids in yeast

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Summary. pKD1 is a 2μ-like circular plasmid found in the yeast *Kluyveromyces drosophilarum* that can also stably replicate in *Kluyveromyces lactis*. We have found a short intergenic region in this genome that appears to be functionally neutral; that is, the introduction of foreign sequences into the single EcoRI restriction site located near one of the inverted repeats did not affect the high stability of the natural plasmid. By introducing a G418 resistance gene at this site, we constructed an autonomous recombinant plasmid. Since this vector did not require *cir*$^+$ hosts for its stable maintenance, it could be used to examine the transformation host range of pKD1 among all the species belonging to the genus *Kluyveromyces*. Both species closely related to *K. drosophilarum* as well as a few other species that are very different in chromosomal GC% could be transformed to yield highly stable transformant clones.

Key words: *Kluyveromyces* – Transformation – 2μ-like plasmids – G418 resistance – pKD1

Introduction

The circular 2μ plasmid of the yeast *Saccharomyces cerevisiae* has been extensively studied by many groups (for review, see Broach 1982). This plasmid encodes a number of proteins that are required for the stable maintenance of the plasmid at high copy number during cell division. The presence of two isomeric forms, generated by internal inversion at the sites of two inverted repeats, is a characteristic feature of this plasmid. The use of recombinant derivatives of 2μ has led to an efficient transformation system for *S. cerevisiae* (Beggs 1978). 2μ-like plasmids that have analogous structures and functional organizations have been found in several other yeast species (Toh-e et al. 1982, 1984; Falcone et al. 1986; Utatsu et al. 1987). Transformation systems equivalent to the 2μ/*S. cerevistae* system have thus been developed for *Zygosaccharomyces rouxii* (Araki et al. 1985) and *Kluyveromyces lactis* Bianchi et al. 1987). The basic principle involved in constructing transforming vectors from the 2μ-type plasmids is to combine their ARS with a marker gene that allows selection of yeast transformants and with bacterial plasmid components that allow amplification of the recombinant molecules in *E. coli*. Since these constructions no longer possess the complete set of genes of the original yeast plasmid, they are defective for stable maintenance in the yeast host. To complement the missing functions, one has to use *cir*$^+$ hosts carrying an intact resident plasmid. A consequence of this situation is that the host specificity of the plasmids with respect to the existing 400 yeast species cannot be directly tested by these recombinant vectors, since it is likely that most yeast species do not possess any resident plasmid that could help in the replication and maintenance of the vector molecules. Furthermore, the selection markers used in *S. cerevisiae* are generally not suitable for the detection of transformants in other yeasts, which are usually prototrophs. For each 2μ-like plasmid the question arises as to its possible use in the transformation of other speceis of interest. This paper proposes an approach to this question.

Our study of the plasmid pKD1, a 2μ-like plasmid found in a strain of *K. drosophilarum,* presented us with the possibility of examining the host range of this plasmid. The principle is the following. In the short intergenic spaces of the plasmid sequence there are several unique restriction sites. One of them showed an exceptional property: insertion of a marker sequence at this site hardly affected the stable maintenance of the

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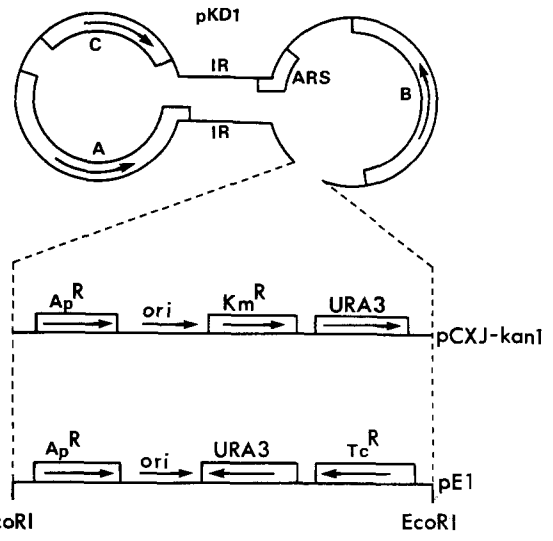

Fig. 1. Structure of pCXJ-kan1 and pE1. pCXJ-kan1 contains the total sequence of pKD1 opened at the unique EcoRI site between the *B* gene and one of the inverted repeats. At this site we inserted the Km^R gene, the *URA3* gene of *S. cerevisiae,* the ColE1 replication origin (*ori*) and the ampicillin resistance gene (Ap^R). An analogous plasmid, pE1, contains the yeast integrative plasmid YIp5 (pBR322 plus *URA3* gene) at the EcoRI site of pKD1. The *A, B* and *C* genes of pKD1, and their direction of transcription are represented, as well as the ARS and the inverted repeats (IR) of pKD1

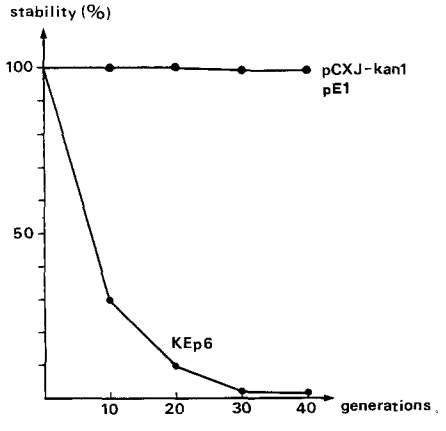

Fig. 2. Stability of *K. lactis* transformants obtained with pCXJ1-kan1. The transformation procedure and transformant selection by either G418 resistance or prototrophy have been described previously (Bianchi et al. 1987; Chen and Fukuhara 1988). The stability of the transformants was determined on single colonies at the percentage of cells that remained resistant to G418 at a concentration of 200 μg/ml (in the case of pCXJ-kan1), or prototrophic for uracil (in the case of pE1 and KEp6) after the indicated number of cell divisions in non-selective liquid medium. The host strain for pCXJ-kan1 and pE1 was MW98-8C (*Matα lysA argA uraA* pKD1° K^+). For comparison, the stabiity of a conventional type plasmid, KEp6, is also shown. This plasmid is constructed by combnining the yeast integrative plasmid YIp5 containing *URA3* with the ARS of pKD1 (Wésolowski-Louvel et al. 1988). In this latter case the host strain was CXJ1-7A (*Matα adel-1 uraA lac4-8* K^+ $pKD1^+$)

plasmid, even in the absence of resident pKD1 in the host (Bianchi et al. 1987). By inserting the kanamycin/G418 resistance gene of the transposon Tn*903* (Km^R) at this site as a universal marker for transformation, we could use this autonomous vector as a direct test for the transformation of various yeast species, since most of them are sensitive to G418. The natural promoter of the Km^R gene is recognized by a number of eukaryotic cells including yeast (Jimenez and Davies 1980).

Results and discussion

Stability of pCXJ-kan1 in K. lactis

The structure of the plasmid pCXJ-kan1 is shown in Fig. 1. The possibility of using this recombinant plasmid for heterospecific transformation was suggested by its replication autonomy and high stabiity in *K. lactis* hosts. Figure 2 illustrates the high stability of this vector in a cir^0 strain (devoid of resident pKD1) over extended periods of time. pE1, another construction of the same type, was also found to be stable under non-selective conditions. For comparison, we show the stability of a vector of a conventional type, KEp6, which carries only the ARS region from pKD1, the pBR322 sequence and the *URA3* gene. In this latter case, a cir^+ host strain was used (otherwise KEp6 is extremely unstable). The stability of this plasmid was comparable to that of 2μ-derived YEp vectors used in cir^+ strains of *S. cerevisiae.*

Transformation frequency and stability of transformants obtained using pCXJ-kan1 and Kluyveromyces species

Representative strains of all available *Kluyveromyces* species were tested for their transformability by pXCJ-kan1. Table 1 summarizes the results of the transformation experiments. Since the optimal conditions for transformation were not established for each species, the absolute values of transformation frequency were not sought; only the order of magnitude was confirmed by repeated experiments. In the case of positive transformation, the stability of the transformants was determined on randomly chosen single colonies.

The autonomous existence of the plasmid in transformed cells was demonstrated by gel electrophoresis of DNA extracted from the transformants and by Southern-blot hybridization. This confirmed that the transformed phenotype was due to the presence of the unmodified input vector and did not result from its integration into chromosomes.

Table 1. Transformation of *Kluyveromyces* species by pCXJ-kan1

Species (GC%)	(Synonym)	Strain[a]	Transformation frequency[b]	Stability of transformants[c]
K. marxianus (39.9–42.6)				
var. *lactis*	(*K. lactis*)	MW98-8C	14,820	99.9
		CBS 141	23,200	91,6
var. *drosophilarum*	(*K. drosophilarum*)	CBS 2105	31,000	98.0
		CBS 2896	40,300	97.1
	(*K. phaseolosporus*)	CBS 2103	19,000	65.5
var. vanudenii	(*K. vandudenii*)	CBS 4372	24,700	85.0
var. *dobzhanskii*	(*K. dobzhanskii*)	CBS 2104	4,500	71.5
var. marxianus	(*K. marxianus*)	CBS 712	167	21.4
		CBS 6556	831	35.1
		CBS 1553	0	
	(*K. fragilis*)	CBS 397	0	
	(*S. fragilis*)	ATCC 12424	0	
	(*K. fragilis*)	ATCC 36907	0	
var. bulgaricus		CBS 2762	0	
var. bulgaricus	(*K. cicerisporus*)	CBS 4857	0	
var. bulgaricus	(*K. wikenii*)	CBS 5671	0	
K. wickerhamii	(42.2)	CBS 2745	447	21.4
K. thermotolerans	(46.2)	CBS 6340	480	68.0
K. waltii	(45.6)	CBS 6430	5,060	97.0
K. aestuarii	(39.9)	CBS 4438	330	80.7
K. polysporus	(35.3)	CBS 2163	*	
K. phaffii	(35.3)	CBS 4417	0	
K. lodderae	(35.5)	CBS 2757	0	
K. delphensis	(40.2)	CBS 2170	0	
K. blattae	(33.1)	CBS 6284	0	
K. africanus	(38.5)	CBS 2517	0	
K. cellobiovorus		CBS 7153	0	
K. yarrowii		CBS 6070	0	
Candida kefir	(41.3)	CBS 1970	0	
Candida macedoniensis		CBS 600	0	
Zygosaccharomyces rouxii	(40.0)	CBS 732	0	
Saccharomyces cerevisiae	(39–41)	S150-2B	54,000	2.6

[a] All the type strains of *Kluyveromyces* species were obtained form Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. A few other strains of independent origin were obtained from The American Type Culture Collection (ATCC), Rockeville, Maryland, USA. The names of species and their genomic GC% were given according to Kreger-van Rij (1984). For the varieties of the species *K. marxianus,* simpler synonyms were used in the text. The two *Candida* strains were included in the test as they are considered to be anamorphs of *K. marxianus* var. marxianus

[b] Transformation was performed as described by Chen and Fukuhara (1988). The transformation frequency was the number of transformants/ 3×10^6 protoplasts per 5 μg plasmid DNA

[c] Stability was percentage of cells retaining the $G418^R$ phenotype after 10 generations in non-selective medium

* Presence of many microcolonies

pKD1 was originally isolated from *K. drosophilarum* CBS 2105 (UCD 51-130) (it therefore contains resident pKD1). The efficient transformation and the high stability of transformants of *K. lactis* strains have been established: the possibility of transferring intact pKD1 plasmid from *K. drosophilarum* to a *K. lactis* strain provided the basis for developing the pKD1-derived vector system (Bianchi et al. 1987).

K. vanudenii, K. dobzhanskii and *K. phaseolosporus* all produced stable transformants at a high frequency. These species have been shown to be closely related to *K. lactis,* as judged from pulsed field gel electrophoresis patterns of chromosomes and by restriction analysis of their mitochondrial DNA (Sor and Fukuhara 1989).

K. aestuarii (a lactose-assimilating species) which can be distinguished from the above group of species by a number of criteria showed a much lower frequency of transformation, although the stability of transformants was surprisingly high. Unexpected results were also obtained with *K. waltii* and *K. thermotolerans.* These two species have a chromosomal GC content of about 46%, suggesting their rather remote evolutionary position with respect to all the above-mentioned species (GC about 40%). The analysis of mitochon-

drial DNA (not presented) also showed patterns that were completely unrelated to those of the *K. drosophilarum-K. lactis* group. Nevertheless, we obtained quite stable transformants with a low, but reproducible frequency of transformation.

Strains of *K. marxianus* and *K. fragilis* gave variable results. Some were transformed, although always at a low frequency, and the transformants were relatively unstable. Such was also the case for *K. wickerhamii.* While this level of stability is probably acceptable for most laboratory experiments, such as gene cloning, it is insufficient for any of the applied purposes for which these species are of particular interest. Various other strains of these species could not be transformed in repeated trials, despite a good regeneration rate of protoplasts. Other procedures of transformation remain to be tested.

We noted that two strains of *K. lactis* (CBS 4574 and 683) failed to produce any transformants. In the case of CBS 4574, the very low regeneration of protoplasts may be the reason. In the case of CBS 683, in which protoplast regeneration was excellent, the "transformation-negative" character seems to segregate through meiosis: when CBS 683 was crossed with a transformation-competent strain (MW98-2B) of *K. lactis,* the meiotic products segregated 1:3 for transformability (4 tetrads analyzed), suggesting the possibility that the competence could be improved by genetic crosses.

S. cerevisiae gave a very high frequency of transformation, but the transformants were all extremely unstable, suggesting that the pKD1-derived vector, while capable of replicating in this host, cannot be correctly partitioned at cell division.

All other species of *Kluyveromyces* showed no transformation. Their chromosome patterns (Sor and Fukuhara 1989) were diverse and quite different from the high transformation group *K. drosophilarum-K. lactis.* The absence of transformation may be due to various reasons: (1) the natural promoter of the Km^R gene cannot be recognized by some species of yeast, although it has always been expressed in the tested cases of yeast, plant and mammalian cells; (2) the standard transformation procedure may be inadequate in some species. Obviously, the presence of negative cases do not exclude the possible transformability of these species and strains. They must be examined individually in more detail.

Conclusion

The finding of a functionally neutral site within the pKD1 genome has led us to construct highly stable, autonomous transformation vectors. The use of such vectors in combination with the G418 resistance marker enabled us to test directly the transformability of many yeast strains. The present survey, limited to the *Kluyveromyces* genus, may be extended to other yeast genera, using the same vector. The presence of intergenic neutral sites is probably not an exceptional property of pKD1, since a preliminary survey suggested the presence of such sites in at least two other yeast circular plasmids (to be published). It should be useful to examine in the similar way the host range of 2μ-derived vectors for stable transformation of a wide range of yeasts. As shown in the above examples, a 2μ-like plasmid may stably replicate in taxonomically unexpected species.

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