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**Inverted terminal repetitions of the two linear DNA associated with the killer character of the yeast *Kluyveromyces lactis***

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**ABSTRACT**

The killer character of some *Kluyveromyces lactis* strains is associated with the presence of two linear double-stranded DNA, pGKI-1 (or k1) and pGKI-2 (or k2). Nucleotide sequencing has revealed that each DNA has inverted terminal repetitions of about 200 base-pairs whose 5' ends seem to be blocked. The repetitions of the two DNA do not share extensive sequence homology. The role of these repetitions in the replication of killer DNA is discussed.

**INTRODUCTION**

Certain strain of the yeast *K. lactis* exhibit a killer phenotype analogous to the killer system of *Saccharomyces cerevisiae* (production of a toxin that kills sensitive yeast cells). While the killer character of *S. cerevisiae* is associated with two double-stranded RNA (see review in ref. 1), the killer system of *K. lactis* is dependent on the presence of two linear double-stranded DNA, called pGKI-1 and pGKI-2 (2), or in this paper k1 and k2 (4) (for full description of the system, see references 2-8). Simultaneous presence of both k1 and k2 in the same cell is necessary for the expression of the killer character. The k1 DNA, 8.8 kilobase-pairs (kbp) long, appears to be directly involved in the toxin production, since several non-killer mutations have been localized within this DNA. The replication of k1 seems to be dependent on the presence of k2 DNA (13.4 kbp), because k2 can replicate in the absence of k1 whilst the converse has never been found. The expression of killer character is also dependent on at least one nuclear gene (4).

Our interest in the interaction between these two plasmids prompted us to further characterize their molecular nature. The replication of these linear genomes is also of general interest because they are one of rare examples of extrachromosomal linear DNA in yeast. Linear genomes from various organisms are known to have unusual terminal structures which are thought to play a role in replication. We therefore first focused our attention to the terminal sequences of killer DNA.

**MATERIALS AND METHODS**

**Nomenclature of killer plasmids.** The two killer DNA, pGKI-1 and pGKI-2 were first

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isolated by Gunge and Sakaguchi (2) from a stock of *K. lactis*, strain IFO 1267. Independently two similar plasmids were found by Wésolowski et al. (4) in the strain CBS 2359 (a stock of Genetic Institute, University of Parma), and called k1 and k2. A detailed comparison, on physiological and physical terms, revealed no difference between these two isolates (4,5,6). According to the ATCC 1982 records, IFO 1267 and CBS 2359 seem to come from the same origin. Another strain CBS 2360, which is different in physiological properties from CBS 2359 (9), harbors also two killer plasmids analogous to k1 and k2 (H. Heslot, personal communication). Therefore pGKI-1 and pGKI-2 appear to be representative of this class of killer plasmids. The names k1 (corresponding to pGKI-1) and k2 (to pGKI-2) are used in this paper for practical reasons.

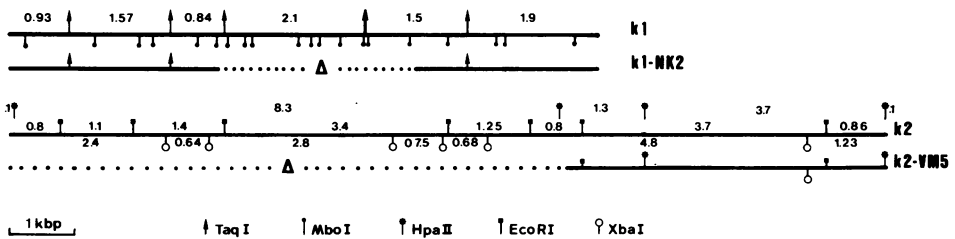
Isolation of plasmid DNA. Our standard killer strain used for plasmid isolation is CBS 2359/152 (a met) (4). The plasmids were extracted and purified according to a published procedure (5). In order to eliminate contamination with mitochondrial DNA, an additional step was included as follows. Each plasmid DNA eluted from preparative agarose gels was dissolved in 10 ml of 1 mM EDTA-Na<sub>2</sub>/10 mM Tris-HCl, pH 8, and mixed with 9.85 g of CsCl and 50  $\mu$ l of 4',6-diamidino-2-phenylindole dihydrochloride (Boehringer, stock solution 10 mg/ml in water). The solution was centrifuged at 38 000 rpm for 48 hours in a Beckman rotor SW50 or 50Ti. The killer DNA banded on top of mitochondrial DNA as a discrete fluorescent band. The DNA was dialyzed, concentrated and kept frozen in 1 mM EDTA/10 mM Tris-HCl, pH 8.

Other techniques. Restriction enzyme digestion, electrophoresis, DNA-DNA hybridization on nitrocellulose paper, nick-translation of DNA and autoradiography, all technical details have been described earlier (10,11). DNA sequencing was carried out according to the chemical method of Maxam and Gilbert (12) with minor modifications. 5' end labelling was performed using bacterial alkaline phosphatase (Bethesda Research Laboratories), T4 polynucleotide kinase (Boehringer) and <sup>32</sup>P- $\gamma$ -ATP (New England Nuclear). 3' end labelling was carried out with terminal deoxynucleotidyl transferase and <sup>32</sup>P- $\alpha$ -cordycepin triphosphate (New England Nuclear) under the conditions recommended by the suppliers.

## RESULTS AND DISCUSSION

### Physical maps of k1 and k2 DNA and properties of terminal regions

Various linear genomes have been shown to have inverted terminal repetitions (for example, adenoviruses, ref. 13, 14 ; ribosomal DNA of *Dictyostelium*, ref. 15 ; macronuclear DNA of ciliates, ref. 16, 17 ; *Tetrahymena* mitochondrial DNA, ref. 34 ; mitochondrial DNA of male sterile maize, ref. 22 ; a plasmid from *Ascobolus*, ref. 18). Terminal repetitions are thought to be involved in some way in the replication of these



**Figure 1** : Physical maps of k1 (8.8 kbp), k2 (13.4 kbp) and their deletion mutants. Upper two horizontal lines show the restriction maps of k1 DNA and its deletion mutant k1-NK2 (5.9 kbp). (Preliminary maps of these genomes have been described in ref. 3). Lower two lines are the maps of k2 DNA and its deletion mutant k2-VM5 (4.9 kbp).  $\Delta$  means deletion of a continuous segment in both mutants. The k2-VM5 map was constructed as explained in Figure 2.

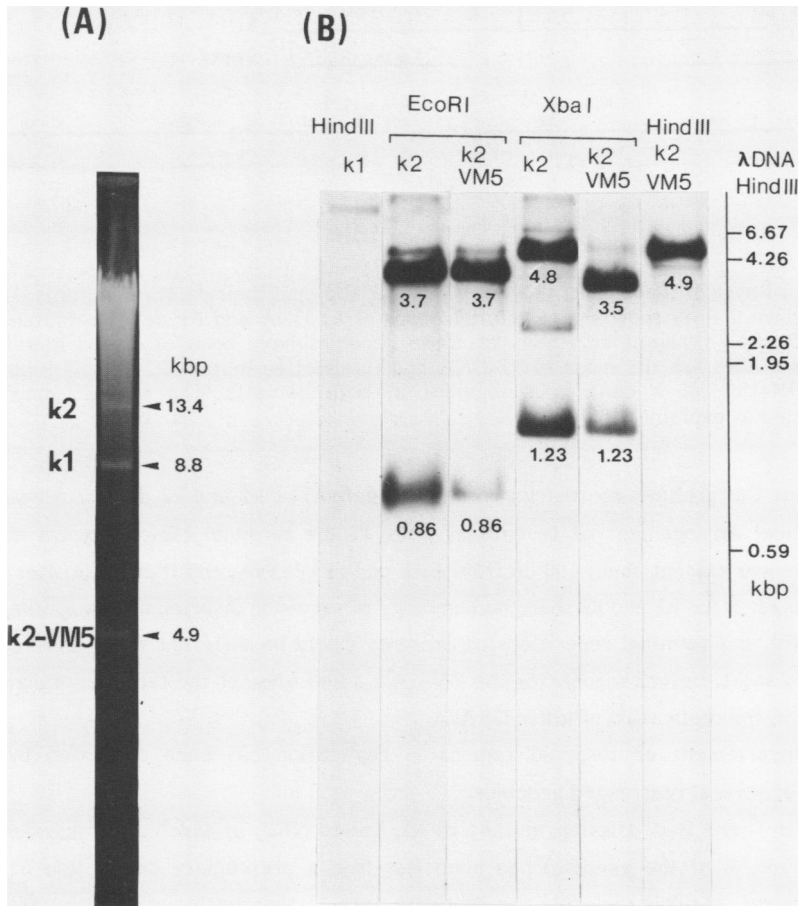
molecules. Our preliminary restriction maps (Figure 1) of k1 and k2 did not reveal any symmetrical arrangement of restriction sites at the terminal regions except that a HpaII site was present about 100 bp from each end of k2 DNA, and that these sites were absent in k1. Since k1 and k2 share no homology of sequence (hybridization experiments, not shown), the terminal repetitions, if present, should be different in k1 and k2, and relatively short. Before sequencing the DNA, we asked whether the terminal regions are required in the replication of killer DNA.

Involvement of terminal regions in replication has been suggested by the analysis of several rearranged genomes.

(i) There is a deletion mutant of k1, called NK2, in which a central 2.9 kbp segment (33 % of the genome) had been lost (see a preliminary description of this mutant in ref. 4). This molecule can replicate with an apparently normal copy number, although the killer activity is lost. This shows that the central segment of k1 is not necessary for replication. A similar mutant has been described by Niwa et al. (8).

(ii) A large deletion mutant has been found also for k2 DNA. A non-killer mutant, called VM5, contained in addition to apparently normal k1 and k2, a new linear DNA of about 4.9 kbp which we call k2-VM5 (Figure 2A). Restriction and hybridization analyses (Figure 1 and Figure 2B) showed that this DNA was a deleted form of the normal k2 DNA with 64 % (8.5 kbp) of the k2 genome deleted from the left end. This case shows that at least the left two thirds of the k2 genome is not required for replication.

(iii) When HindIII digested k1 DNA was randomly ligated to a pBR322 plasmid carrying the *URA3* gene of *S. cerevisiae*, some of the recombinant plasmids were able to transform a uracil requiring *K. lactis* strain (*uraA*) into prototrophic colonies ; a few such plasmids have been characterized and found to contain always either one or both



**Figure 2** : Structure of the deletion mutant k2-VM5. (Left) : Normal killer strains contain only k1 and k2 plasmids. A non-killer mutant VM5 showed, in addition to k1 and k2, a third plasmid named k2-VM5. Total DNA of the mutant was extracted and electrophoresed in a 0.6 % agarose gel and visualized by ethidium bromide staining. (Right) : k1, k2 and k2-VM5 DNA were digested with restriction enzymes as indicated, electrophoresed on 1.5 % agarose, and "southern transfers" on nitrocellulose filters were prepared. The filter-bound DNA was hybridized with <sup>32</sup>P-labelled k2-VM5 (nick-translation). k1 DNA digests did not show a significant hybridization with the k2-VM5 probe (lane 1). In contrast, the probe hybridized with a few specific fragments of k2 DNA, and only those fragments were detected by autoradiography as shown (all the fragments of k2-VM5 DNA were detected, as expected, under these conditions). The indicated fragment sizes are to be compared to the k2 DNA restriction map of Figure 1. Note that k2-VM5 probe detected only EcoRI 3.7 and 0.86 kbp fragments (lane 2) in k2 and the same bands were present in the mutant (lane 3). The probe detected also XbaI 4.8 and 1.23 kbp fragments of k2 (lane 4), but, in the mutant, XbaI 4.8 was replaced by a fragment of 3.5 kbp while XbaI 1.23 was conserved (lane 5). Undigested k2-VM5 (no HindIII site) gave a unique band of 4.9 kbp (lane 6), a size in agreement with the sum of digested fragments of k2-VM5 shown in lanes 3 and 5. This example is one of the hybridization experiments from which the k2-VM5 map of Figure 1 was deduced.

of the terminal segments (1.0 to 2.3 kbp) of k1 (19). These segments therefore should contain an element capable of supporting replication of the bacterial plasmid in yeast.

The 5' ends of k1 and k2 DNA are not accessible for phosphorylation in vitro

Some linear DNA genomes have their 5' termini in a form inaccessible for phosphorylation by polynucleotide kinase even after treatment with bacterial alkaline phosphatase. A typical case of this kind has been described in a careful study of adenoviruses by Carusi (20). Rekosh et al. (21) have demonstrated that the blocking element was a protein covalently attached to the 5' terminal nucleotide of the adenovirus DNA. An analogous example has been reported for the mitochondrial S1 and S2 DNA associated with a male sterile strain of maize (22). As in these cases, pronase treated killer DNA was still resistant to phosphorylation even after denaturation at elevated temperature. The exact nature of the blocked 5' end is currently under investigation. It is to be noted that another cytoplasmic linear DNA of yeast, the mitochondrial DNA of *Hansenula mrakii* (23) has quite different 5' ends which can be phosphorylated under our standard conditions using T4 polynucleotide kinase. Thus, two kinds of linear DNA can replicate in yeast, one with blocked 5' ends, another non-blocked, which should reflect the presence of different systems for 5' priming reaction of linear DNA synthesis. The 3' ends of k1 and k2 DNA appear to have free hydroxyl termini because labelled nucleotides can be attached to them by terminal deoxyribonucleotidyl transferase, and this reaction was used for sequencing these DNA from their 3' ends.

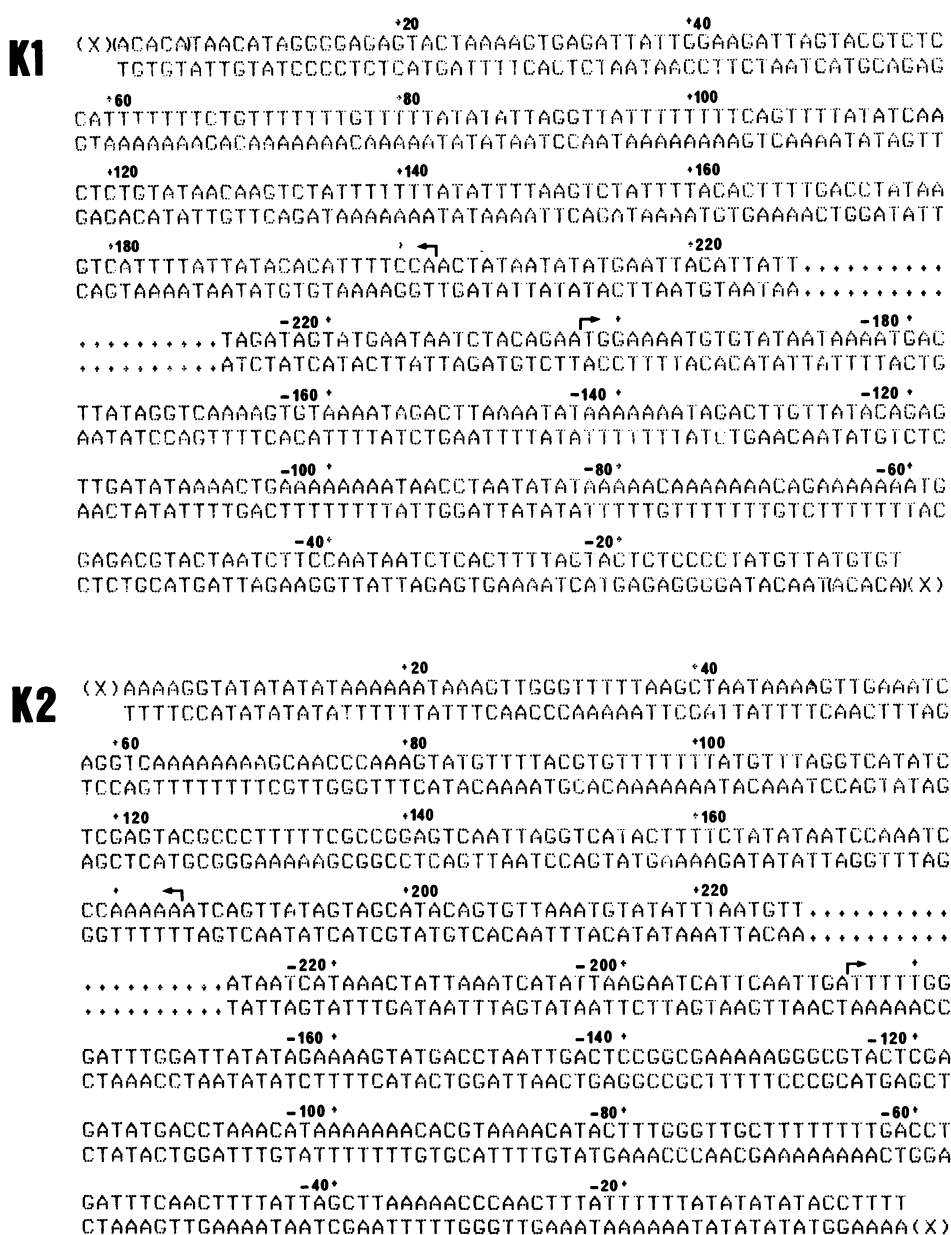
Nucleotide sequence of the terminal regions

The terminal regions of k1 and k2 DNA were sequenced on both strands either from the free 3' ends or from internal restriction sites labelled at 5' or 3'. Figure 3 shows the sequences of about 250 bp of the left and the right ends. The last few nucleotides of the 5' ends are not established because of their blocked structure. The salient features of these terminal sequences are described as follows.

(i) In k1 DNA, 202 bp of the left end sequence is precisely inverted at the right end of the molecule. Similarly, k2 DNA has also inverted terminal repetitions of 184 bp long. The size of these repetitions is comparable to those found in adenoviruses (14), or S1 and S2 mitochondrial DNA of maize (22). The inverted terminal repetitions of k1 and k2 DNA have no obvious sequence homology between them.

(ii) No suggestions of sequence discontinuities, or gaps, were observed during sequencing studies, neither in k1 nor in k2. Single strand discontinuities are known to exist in some linear genomes (24).

(iii) Tandemly repeated simple sequences of the type  $C_4A_4$  (25,26,27),  $C_4A_2$  (23,28,29),  $C_nT$  (30) or  $(GT)_n$  (31) are not seen in k1 and k2 DNA. These tandem repetitions characterize the terminal regions of some linear DNA.



**Figure 3** : Sequences of the terminal regions of k1 and k2 DNA. The left and right terminal regions of k1 and k2 DNA are shown. Discontinuous lines represent the central part of each genome. Terminal inverted repeats are marked by arrows. 5' ends of upper strands are placed to the left. The very last nucleotides of 5' ends are not known. Nucleotides in parentheses were deduced from the experimentally determined sequence of the opposite strand. (X) means possible presence of 5' protruding nucleotide(s).

(iv) The termini of k1 and k2 do not contain an internal palindromic sequence. The presence of terminal palindromes has been proposed by Cavalier-Smith (32) and Bateman (33) to solve the problem of 5' end filling in the synthesis of linear DNA. The blocked nature of the 5' ends of killer DNA may suggest, in fact, involvement of a terminal protein in 5' end synthesis (see review by Wimmer, ref. 35).

(v) The terminal repetitions contain several short dispersed repeats, either direct or inverted, and can be folded into internally base-paired structures. k1 and k2 are quite different, not only in primary sequences but also in folded forms. A feature that seems to be common for the two DNA is the fact that the 5' strands (upper strands) of the terminal repetitions are rich in purines (62 % over 60 bases in k1, and 67 % over 80 bases in k2) followed by a sequence relatively rich in pyrimidines. The high adenine and thymine contents of the inverted terminal repetitions (76 % for k1 and 79 % for k2) are not peculiar to the repeats since these values are close to the overall composition of the killer plasmids.

Among fungi, some strains of Ascobolus immersus have been reported to contain a linear DNA plasmid of about 6 kbp. This plasmid also seems to have inverted terminal repetitions as judged by electron-microscopic studies (18).

The inverted terminal repetitions are frequent in the linear DNA of animal, plant or viral origins. Fungal plasmid DNAs now offer a new tool for examining the function of these repetitions.

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