A palindromic mutation of the linear killer plasmid k2 of yeast

Micheline Wésolowski-Louvel* and Hiroshi Fukuhara

Institut Curie, Section de Biologie, Centre Universitaire, 91405 Orsay, France

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ABSTRACT

Production of the killer toxin in *Kluyveromyces lactis* is dependent on the presence of two linear DNA plasmids, k1 and k2. We isolated a non-killer mutant, VM5, with a modified plasmid composition. It had lost k1, but conserved k2, and acquired, in addition, three new DNA species. The new species were found to be rearranged derivatives of the k2 plasmid. One of them, pVM5-1, was made of the left terminal 4720 bp sequence of k2, including the inverted terminal repeat, and was organized as a large palindromic dimer molecule. The second, pVM5-2, was made of one strand of the pVM5-1 palindrome, folded into a hairpin structure. Like normal k2, pVM5-1 and 2 were present in a high copy number. The third species, pVM5-x, of variable size, was also a deletion product of k2, but not palindromic, and did not contain the terminal repeat. Genetic analysis showed that the presence of the palindromic derivatives appeared to destabilize the normal k2 genome, leading to gradual accumulation of plasmid-less cells.

INTRODUCTION

Killer strains of the yeast *Kluyveromyces lactis* secrete a protein toxin, production of which is associated with the presence of two linear double-stranded DNAs, k1 and k2 (or pGKL-1 and 2 respectively) (1,2). Both plasmids have inverted terminal repeats (3,4), and their 5' ends are blocked by an attached protein, which is characteristic of protein-primed DNA replication systems identified in ϕ 29 and adenovirus. The complete nucleotide sequences of k1 (8874 bp) and k2 (13457 bp) have been determined (5,6,7,8). k1 codes for the killer toxin subunits, for an immunity determinant and its own DNA polymerase (9). k2 which codes for its own DNA polymerase, a RNA polymerase and an helicase (8,10) is thought to have essential functions for the maintenance of k1.

The search for plasmid mutations affecting a specific function has been hampered by a high frequency of plasmid loss in induced mutagenesis. Except in cases of total loss of both plasmids, most of the reported mutations concern k1 (Table 1). The gene organization of the k1 genome is largely understood at present. In contrast, our knowledge of the nature of the k2-encoded genes

is limited to the features deduced from nucleotide sequence data.

Among the plasmid mutations we have isolated, we describe here one mutation that occurred in the plasmid k2. The mutant strain contained k2-derived palindromic molecules which can be maintained only in the presence of the wild type k2. Analogous deletion mutants leading to palindromic derivatives have been known for the plasmid k1 (11). The study of such derivatives has led to a model of replication of this linear plasmid, involving a strain displacement mechanism. The present study on the plasmid k2 suggests that the model also applies to this genome.

Since k1 is dependent on k2, a possible use of k1 derivatives as a gene vector had been considered (12). Existence of large deletion derivatives of k2 suggests that these forms may also be used as a tool for gene transfer.

MATERIALS AND METHODS

Strains and media

VM5 is a non-killer mutant isolated from the strain MW26-3D (α *lysA*, K^+ , R^+) by UV mutagenesis (1). Culture media and genetic techniques have also been described.

Test of killer phenotype

The plate assay of the killer phenotype was as previously described (1). In some cases, Bactoagar Difco was replaced by Oxoid no.1 agar which considerably increased the sensitivity of the assay (13).

Rapid colony analysis of killer plasmids

Rapid analysis of killer plasmids in many individual clones was performed as follows. Individual colonies were taken up with a toothpick and suspended in 20 μ l of Zymolyase solution (Zymolyase 5T, Kirin Brewries, Tokyo, 6 mg/ml in 50 mM Tris-HCl/ 5 mM EDTA, pH 8) in a Eppendorf microtube. The mixture was incubated at 37°C for 30 minutes. After the addition of 1 μ l of 5% sodium dodecylsulfate and 5 μ l of proteinase K (2 mg/ml), incubation was continued for 1 hour. The lysate was mixed with 5 μ l of marker dye solution, and 20 μ l was used for electrophoresis (0.6% agarose gel). DNA was detected by ethidium bromide staining. With minor modifications, using reduced amounts of enzymes, the procedure was also used for plasmid DNA purification.

* To whom correspondence should be addressed

Manipulations of DNA

The palindromic junction fragment in pVM5-1 could not be cloned when we used usual *E. coli* host strains (RR1, HB101, etc.), probably because of its palindromic nature. The fragment was finally cloned in a *recBC sbcB recA* strain, DL 283, and sequenced by the chemical method of Maxam and Gilbert (14). All other techniques were those described in standard laboratory manuals.

RESULTS AND DISCUSSION

Origin of the mutant VM5

A preliminary description of this mutant was given in 1982 (1). It was isolated as a non-killer mutant after a ultraviolet mutagenesis. When we first examined the plasmid composition of this mutant by electrophoresis, k1 and k2 seemed normal, but,

Figure 1. Detection of plasmids in the mutant VM5. Analytical gel electrophoresis. The mutant and wild type cells were analyzed by the rapid method described in Materials and Methods. (A) A typical plasmid pattern of VM5 (slot 1) and the wild type killer strain (slot 2). (B) Examples of subclones from VM5. Note, in the first and second lanes, the faint band of pVM5-X, and in the third lane, the loss of pVM5-1 and pVM5-2 leaving only k2.

in addition, there were new smaller components. It was not understood why this mutant was non-killer and sensitive to the toxin despite the apparent presence of both k1 and k2; the genetic analysis of the mutant plasmids met also some difficulties. By re-examining VM5 we found, as shown below, that the mutant contained a normal k2 plasmid and its deletion derivatives, but not the k1 plasmid (one of the k2 derivatives had been taken for k1). The difficulties of previous genetic analysis were explained by a genetic instability of the VM5 plasmids.

Plasmid composition of the non-killer mutant VM5

Electrophoresis of the mutant DNA revealed the presence of three major bands (Figure 1A). One was the normal k2 molecule, as this was verified by restriction analysis. k1 was absent. The second component, called pVM5-1, showed a molecular weight of about 9.4 kilobase-pairs (kbp), migrating slightly slower than the reference k1, 8.8 kbp. The third component, pVM5-2, had an apparent size of about 4.7 kbp. On molar basis, each of pVM5-1 and pVM5-2 seems to have a several-fold higher copy number with respect to k2 (which, in normal killer strains, has a copy number of 50–100 per cell). In some subclones of the VM5 mutant, we noticed the presence of an additional minor component (pVM5-X), visible as a faint band on the stained gel. The size of this component varied considerably (4.1 to 10 kbp) depending on the clone (Figure 1B). In order to identify the origin of the three new DNA species, these bands were transferred to nitrocellulose filters and hybridized with either radioactive k1 or k2 prepared from the wild type killer strain (Figure 2). While the k2 probe strongly hybridized with all of the three new DNAs, the k1 probe hybridized with none of them. Therefore, pVM5-1, pVM5-2 and pVM5-X should derive from the k2 plasmid.

Structure of pVM5-1

Restriction enzyme analysis of pVM5-1 revealed that this plasmid contained only the left 35% of the wild type k2 sequence, including the terminal repeat. The sum of the molecular weight

Figure 2. Origin of VM5-1 and VM5-2 sequences. Wild type (lane 1) and mutant (lanes 2 and 3) DNAs were electrophoresed and the plasmids visualized by ethidium staining. They were then transferred onto a nitrocellulose sheet and hybridized with $\alpha^{32}\text{P}$ -labelled probes: hybrids were detected by autoradiography (lanes 1', 2' and 3'); the probes were: A) entire k1; B) entire k2, or 3.6 kbp *Xba*I fragment of pVM5-1 which corresponds to the 4.8 kbp fragment of k2 (see figure 5).

of restriction fragments corresponded to about one half of the size estimated by electrophoresis of undigested pVM5-1. The discrepancy can be explained if the molecule formed a repetitive structure. A palindromic dimer was a possible candidate, since the linear molecule should have inverted repeats at the two

a) Sequence of the 350 bp EcoRI fragment of pVM5-1

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GAATTCGTATATTTTATCTTCTTCTAACTGTTTCATTAA
EcoRI
ATTTTTAGGACTTCTTTCAGAAAATTCTTCATTTTCTA
ATATAAATATCCATTTTGTATCTTAAATCTACTGTAT
TTTTTGAATTTAGATTTATATATACTTTTTTCTTATT
CTTTATAT[TGGTTAT]ATA[ATAACCA]ATATAAGAATAG
GAAAAAA...
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b) Sequence of the 760bp EcoRI fragment of K2

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4554
GAATTCGTATATTTTATCTTCTTCTAACTGTTTCATTAA
EcoRI
ACATTAACATTTTTAGGACTTCTTCTGAAAATTCTTC
ATTTTCTAATAATAAATATCCATTTTGTATCTTAATC
TACTATTTTGAATTTAGATTTATATACTTTTTTCTTA
TTCTTTATAT[TGGTTAT]TAT[ATAACCA]TCTGTTTCAA
4745
ATCATGC...
```

Figure 3. Nucleotide sequence of the central junction in the palindromic pVM5-1 genome. The central 350 bp EcoRI fragment of pVM5-1 (see Fig. 5) and the 760 bp EcoRI fragment of the k2 wild type plasmid were cloned into pBR328, before sequencing. DNA fragments were labeled at the 5' ends with γ - 32 P ATP and polynucleotide kinase. Nucleotide sequences were determined according to Maxam and Gilbert (14). The boxed sequences are the inverted heptanucleotides found in the wild-type genome. Numbers at the top of the nucleotide sequence of the k2 fragment are the positions at which they are found in the k2 genome sequence (8).

Figure 4. Denaturation analysis of pVM5-1, 2 and 3. (A) Purified pVM5-1 (lanes 1 and 2), pVM5-2 (lanes 3 and 4) and pVM5-3 (lanes 5 and 6) were alkali-denatured, renatured and electrophoresed in a neutral gel (even numbers); odd numbers: untreated plasmids. (B) pVM5-1 (lane 8) and pVM5-2 (lane 9) were alkali-denatured and electrophoresed in an alkaline gel.

termini. We therefore determined the nucleotide sequence corresponding to the region of the deletion point, near the central EcoRI sites, in the pVM5-1 as well as in the corresponding region from the wild type k2 (Figure 3). We found that this central segment indeed contained the junction of large inverted sequences.

The symmetry was nearly perfect, with a short trinucleotide ATA at the center (or TAT for the other strand). It is to be noted that, in the wild type genome, this unique trinucleotide was flanked on both side by a pair of inverted heptanucleotides TGGTTAT TAT ATAACCA which may be involved in the deletion mechanism (15). Presence of a central junction segment of variable length is a typical feature found in other well studied palindromic genomes such as the extrachromosomal rDNA of *Tetrahymena* (16) or *rho*⁻ mitochondrial DNA of yeast (15).

Table 1. List of mutations* of the killer system

Name of mutant plasmid	Characteristics	Reference
<i>Mutations of k1:</i>		
k1-NK2	Central 2.9 kbp deletion in ORF2; k2 present; non-killer.	1, 23
pGKL1S	Analogous to NK2; k2 present; non-killer	24
F1/F2	Palindrome, 5 kbp right terminal deletion; k2 present; non-killer.	11
pK192L/pK192S	Palindrome, 6.5 kbp right terminal deletion; k1 and k2 present; weak killer.	25
k1-H82	Point mutation in ORF2 of k1; k2 present; non-killer.	1,22
k1-VA3	Point mutation in ORF2 of k1; recombines with H82; k2 present; non-killer.	1,22
Total loss	Frequent; k2 either present or absent; non killer.	1,24
<i>Mutations of k2:</i>		
pVM5-1/pVM5-2	Palindrome, 8.7 kbp right terminal deletion; k2 present, k1 is lost; non-killer.	1 and this work
pG21Y1/Y2	Palindrome, 5.4 kbp right terminal deletion; k2 present, k1 present; killer.	17
pG2T1/T2	Palindrome, 6.4 kbp left terminal deletion; k2 present, k1 present; killer.	17
Total loss	Frequent; k1 absent; non-killer.	1,24
<i>Host mutations:</i>		
kex1	All non-killer host mutations (a few hundreds) mapped to this chromosomal locus coding for a toxin processing proteinase; recessive; homozygous diploids are defective in sporulation.	1,26,27

*Plasmid mutations are indicated by the name of the modified plasmid. The host mutation is given by the name of the locus.

Figure 5. Structures of pVM5-1 and pVM5-2. The restriction map and the organization of the wild type k2 genome are shown at the top. The right 65% of the genome is deleted and the remainder is inversely duplicated in pVM5-1. pVM5-2 is made of one strand of pVM5-1, folded into a hairpin form. An open circle represents the terminal protein attached to the 5' end of each strand. ORFs 2, 4 and 6 probably encode for a DNA polymerase (8), an helicase (10,28) and a RNA polymerase (28), respectively. Restriction sites : B : *Bam*HI, R : *Eco*RI, X : *Xba*I, H : *Hpa*II. Nucleotide sequence of the central junction in the palindromic pVM5-1 genome and the wild type sequence at the deletion point are also shown.

Structure of pVM5-2

pVM5-2 molecule showed the following properties : (i) the restriction map of the molecule was very similar to pVM5-1; (ii) its apparent length was that corresponding to the hemimere length of the palindrome (Fig. 1); (iii) when pVM5-2 was denatured and electrophoresed in alkaline gels, it migrated exactly like denatured pVM5-1 (Fig. 4B); (iv) when pVM5-1 was denatured and re-natured, it migrated exactly like pVM5-2 in a neutral gel (Fig. 4A). These observations indicated that the single strand length of the two plasmids was similar, and pVM5-2 can be obtained from pVM5-1. This type of molecules has first been described for a deletion mutation of the k1 genome (11). A palindromic molecule was accompanied by a second molecule of one half size. It has been demonstrated that the latter component was made of one strand of the palindrome, folded into a hairpin structure. The above denaturation experiments indicate that pVM5-2 is also a hairpin derivative of pVM5-1, suggesting that Kikuchi et al's model for k1 (11) also applies to the present case of k2 mutation, that is, pVM5-2 may be a replication intermediate of pVM5-1. The k2 deletion mutations found in *S. cerevisiae* by Gunge and Kitada (17) appear to be of the same nature. Thus deletion, rather than point mutations, seems to be a frequent form of mutation in the linear killer plasmids, as we can see in Table 1. The *rho*⁻ deletion mutation of *S. cerevisiae* mitochondrial DNA is also a well known high frequency event, and very often leads to the formation of palindromes (15).

The structures of pVM5-1 and 2 are schematically shown in Figure 5. The palindromic structure of the pVM5-1 genome included the 184 bp terminal inverted repeats of the original k2. In pVM5-2, one end of the double-stranded molecule is supposed to be a single-stranded loop structure made of the central asymmetric sequence of the pVM5-1 palindrome.

Structure of pVM5-X

Compared to the above two plasmids, pVM5-X was present only in about 5% of the subclones of VM5. In those subclones, pVM5-X appeared as a faint band of variable size. Because of its low amount, its variable size and its relatively frequent loss, the analysis of pVM5-X presented difficulties. Only limited information has been obtained on one such components, 4.1 kbp long, called pVM5-3 : (i) Hybridization with labelled k2 fragments has shown that pVM5-3 contained the left third of the k2 genome, as in the case of pVM5-1 and 2 ; (ii) pVM5-3 did not contain the inverted terminal repeat of k2, since labelled pVM5-3 did not hybridize to the terminal repeat fragment; (iii) pVM5-3 is not palindromic, because when it was alkali-denatured, renatured and electrophoresed in a neutral gel, we could not recover any DNA detectable by ethidium staining, under the condition where pVM5-1 and 2 could reform the double-stranded hairpin structure (Figure 4A). The wild type k2 did not renature under the same condition. Therefore, pVM5-3 has not a palindromic structure and is likely not circular. The presence of pVM5-X is not necessary for the maintenance of

Table 2. Genetic analysis of VM5

Cross partner	Killer phenotype	Plasmids ^a k1/k2	Diploid phenotype when crossed to VM5 (k ⁻)	Plasmids of diploid ^c	Meiotic segregation Killer	Plasmids ^c	Number of tetrads analyzed
2359/3 <i>a hisA</i>	K ⁻	-/-	K ⁻	k2/pVM5-1/pVM5-2	4K ⁻ :0	4(k2/pVM5-1/pVM5-2):0	5
MW109-3D <i>a uraA trpA</i>	K ⁻	-/+	K ⁻	k2/pVM5-1/pVM5-2	4K ⁻ :0	4(k2/pVM5-1/pVM5-2):0 4(-/-):0	4 1
1267/20 <i>a trpA</i>	K ⁺	+/+	K ⁺	k1/k2/pVM5-1/pVM5-2	4K ⁺ :0 4K ⁻ :0 K ⁺ b:K ⁻	4(k1/k2/pVM5-1/pVM5-2):0 4(k2/pVM5-1/pVM5-2/pVM5-x):0 (-/-/-):0	2 1 2

^a presence (+); absence (-)^b in one tetrad tested two spores were K⁻, one spore weak killer, and one spore was divided into K⁺ and K⁻ sectors^c presence of pVM5-X was not recorded

pVM5-1 and 2. pVM5-X may be thought to be a transient product of VM5 genome, in view of the destabilization of the k2 DNA itself in this mutant (see below).

Genetic properties of VM5

Frequency of transmission of VM5 genome in genetic crosses
VM5 strain was crossed with strains having different killer plasmids combination. Results are summarized in Table 2. Plasmid composition of 5 complete tetrads were determined for each cross. The k2/pVM5-1/pVM5-2 plasmids were transmitted in a non-Mendelian manner, as are for k1 and k2 plasmids. The heterogeneity of the tetrads in terms of plasmid composition probably reflects the instability of the VM5 genome (see later). The absence of k1 in the original VM5 mutant is likely not related with the k2 deletion event. Rather, the loss may be a secondary event. The replication of k1 plasmid is compatible with the presence of pVM5-1 and pVM5-2 (Table 2).

Co-segregation of pVM5-1 and 2, and the necessity of the presence of k2

pVM5-1 and pVM5-2 were not found segregating from each another, and were always associated with the k2 plasmid.

Each of k1 and k2 is thought to use its own DNA polymerase for replication: k1 ORF1 product and k2 ORF2 product are putative DNA polymerases (8,18,19,20,21). Besides the DNA polymerases, all other components necessary for the maintenance of k1 and k2 should come from the k2 genome and the host chromosomes. Among the ten ORFs of the k2 genome, as established by Tommasino et al. (1988), only ORF1, ORF2, a part of ORF3, and the terminal repeat are retained in pVM5-1 and 2, the remaining seven being deleted. Since the maintenance of pVM5-1 and pVM5-2 requires the presence of the wild-type k2 plasmid, the latter should provide some trans-acting functions of ORF3 to ORF10 for the replication of the deletion derivatives.

Presence of the pVM5-1 and 2 destabilizes k2

pVM5-1 and pVM5-2 tend to be lost during prolonged culture. A purified clone having a representative plasmid composition was grown for about 80 generations on a complete medium, subcloned (additional 20 generations) and plasmid composition was examined in 28 subclones. We found that 20 conserved the original pattern, 5 contained only k2, and 3 lost all the plasmids.

The palindromic derivatives, pVM5-1 and 2 are thus

preferentially lost. It is interesting to note that there is also a very frequent loss of k2 and/or k1 in the case of crosses of VM5 with a killer strain described above. A cross with VM5 is in fact an efficient way of curing the killer strains. The strains carrying k1 and k2 or carrying only k2 usually do not lose the plasmids under normal conditions of growth. The spontaneous loss of k2 in the VM5 strain should be related with the presence of the palindromic derivatives which may be mutagenic. For example, recombination between the palindromes and k2 may destabilize the plasmid population. Recombination is known to occur between homologous killer plasmids (22). We expect that k2 should not be destabilized by the presence of k1-derived palindromes. Indeed, inspection of the data reported by Kitada and Gungé (1988) clearly shows that the presence of palindromic derivatives of k1 leads to a specific loss of k1 at a high frequency, but k2 remains stable. Another reason for instability may be a possible competition between homologous derivatives for the components required for replication, such as the terminal proteins.

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