Genetic instability of an oligomycin resistance mutation in yeast is associated with an amplification of a mitochondrial DNA segment

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ABSTRACT

In the yeast *Kluyveromyces lactis*, mutations affecting mitochondrial functions are often highly unstable. In order to understand the basis of this genetic instability, we examined the case of an oligomycin resistant mutant. When the mutant was grown in the absence of the drug, the resistance was rapidly lost. This character showed a typical cytoplasmic inheritance. The unstable resistance was found to be associated with the presence of a repetitive DNA in which the repeating unit was a specific segment of the mitochondrial DNA. The amplified molecules were co-replicating with the wild type genome in the mutant cells. The spontaneous loss of the drug resistance was accompanied by the disappearance of the amplified DNA. The repetitive sequence came from a 405 base-pair segment immediately downstream of a cluster of two transfer RNA genes (threonyl 2 and glutamyl). Modified processing of these tRNAs was detected in the mutant. A possible mechanism by which these events could lead to drug resistance is discussed.

INTRODUCTION

In many yeast species, including *Kluyveromyces lactis*, the respiratory function is not essential for survival. Although the *rho*⁻ type mitochondrial mutation is not known in *K. lactis*, several kinds of mutations that affect mitochondrial functions have been described (1, 2, 3, 4). When we analyzed these early works, it appeared that many of these mutations were very unstable, especially in the case of drug resistance mutations. As this aspect had not been discussed in these reports, we re-examined some of the experiments, and found that in fact many of the drug resistance mutations had a very high rate of spontaneous reversion. In order to understand the origins of these genetic instabilities, we investigated the case of an oligomycin resistance mutation.

MATERIALS AND METHODS

Strains. The *K. lactis* strain 2360/7 (*alpha lysA*), obtained from A.Algeri (University of Parma), is a lysine requiring mutant of the strain CBS 2360 (Centraalbureau voor Schimmelcultures, Delft). All the oligomycin resistant mutants (or1 to or6) were obtained from 2360/7. The strain used for the genetic crosses with the mutants was 2359/152 (*a metA*) isolated from CBS 2359.

Growth media. Glucose complete medium contained glucose 2%, Yeast extract Difco 1%, and Bactopeptone Difco 1%, buffered at pH6.5 with 50 mM sodium phosphate. Glycerol complete medium contained glycerol 2% in place of glucose in the above medium. The oligomycin medium was the complete glycerol medium supplemented with oligomycin (Sigma Chemical Co., St.Louis, no.0-1295 or 4876) 3 µg/ml. For plate culture, these media were supplemented with Bactoagar Difco at 2.3%. The minimal medium used for

Table 1. Instability of oligomycin resistant mutants.

Mutant	Frequency of oligomycin resistant colonies (%) after 10 generations of growth in glucose complete medium:	
	primary clone	secondary clone
or1	5	0
or2	35	42
or3	8	10
or4	20	15
or5	22	3
or6	20	11
or3*	—	100

Each mutant clone (primary clone) was grown in a glycerol complete liquid medium for 24 hours, then plated on glycerol complete agar with or without oligomycin. Percentage of resistant colonies was scored. One of such resistant clones (secondary clone) was randomly taken from each plate and tested again for its stability. About 300 colonies were counted for each determination. The strain or3* is one of the faster growing subcolonies which appeared on the surface of the resistant colonies after 2 weeks incubation of the or3 mutant on drug-containing plates.

auxotrophic tests contained Yeast Nitrogen Base without aminoacids Difco 0.67% and Bactoagar 2.3%. Genetic crosses and sporulation were made on ME plates (malt extract Difco 5%, Bactoagar 3%). Tetrad analysis was performed as described previously (5). *Isolation of mitochondrial DNA (mtDNA)*. Cells were grown in one litre of complete medium containing ethanol 2%, yeast extract 1%, Bactopeptone 1%, and sodium phosphate buffer 50 mM, pH 6.5, with or without oligomycin (3 μ g/ml). They were harvested at the stationary phase of growth (2 days shake culture at 28°C), washed in cold water, and suspended in 40 ml of 5 $\times$ TE (1 $\times$ TE is Tris-HCl 10 mM, pH 8, EDTA 1 mM). The ice-cold suspension was mixed with 20 g of glass beads (diameter 0.5 mm, Braun, Melsungen), and cells were mechanically broken in a Braun shaker for 1 minute at the top setting. Cell debris and glass beads were removed by centrifugation at 3 000 rpm for 10 min (Sorvall rotor SS34). The supernatant was adjusted to 1% sodium sarcosinate, and shaken with one volume of water-saturated phenol for 15 min. After centrifugation at 5 000 rpm for 20 min, the aqueous phase was collected and precipitated with 1.5 volume of ethanol after the addition of one tenth volume of 3 M sodium acetate. The DNA precipitates were collected by centrifugation, washed once with 70% ethanol and dried. The DNA, dissolved in 18 ml of 5 $\times$ TE, was mixed with 18 g of CsCl and 100 μ l of bisbenzimidazole solution (Hoechst 33258, stock solution 10 mg/ml). The mixture was centrifuged at 60 000 rpm for 6 hrs in a Beckman rotor VT8. The upper of the two fluorescent bands (mtDNA) was purified by a second CsCl-bisbenzimidazole centrifugation. The dye was removed from the DNA by shaking with isopropanol saturated with 4M CsCl. The DNA was concentrated and dialyzed against 0.1 $\times$ TE.

Manipulation of DNA. The procedures for restriction analysis of DNA, electrophoresis, labelling and filter hybridization were those described in standard laboratory manuals. DNA was sequenced by the dideoxy chain termination method using commercial kit reagents.

Preparation of mitochondrial RNA (mtRNA). Protoplasts were prepared from cells grown as above, and mitochondria were purified by differential centrifugation. RNA was extracted according to Faye et al. (6) with minor modifications.

Analysis of mtRNA. Electrophoresis of mtRNA was performed in 1.5% agarose gels

Figure 1. Restriction patterns of the oligomycin resistant mutant or3. MtDNA from the or3 mutant and the wild type strain was digested with the indicated enzymes, and electrophoresed in 1% agarose gels. Gels were stained with 0.5% ethidium bromide.

containing 6M urea, using Tris-phosphate buffer at 0°C as described by Locker et al. (7). RNA was detected by ethidium bromide staining. For hybridization experiments, RNA was transferred from the gel to Hybond nylon sheets (Amersham) using 3M NaCl/ 0.3M Na₃-citrate.

RESULTS

The unstable oligomycin resistance and its cytoplasmic inheritance.

Oligomycin is a potent inhibitor of mitochondrial ATPase. Most yeasts cannot grow on respiratory substrates when this drug is present. Oligomycin-resistant mutants arose spontaneously on oligomycin-containing agar plates (glycerol complete medium) with a relatively high frequency of the order of 10^{-5} . We randomly selected 6 such clones, which were designated or1 to or6. Each of them was subcloned on drug-containing medium and a single subclone from each primary isolate was retained for further study. In order to determine their genetic stability, these subclones were grown in liquid complete glucose medium for 24 hours (about 10 doublings), then cells were plated on glycerol complete

Figure 2. Origin of the excess DNA in the or3 mutant. DNA was isolated from the mitochondria of the or3 mutant and of the wild type strains, and digested with the indicated enzymes. After electrophoresis, the DNA was transferred to nitrocellulose paper for hybridization. The ³²P labelled probe was the 400 bp HaeIII fragment of the or3 DNA, isolated from a separate gel. Hybridization condition was 0.6 M Na⁺, 58°C, 18 hours. Hybrids were detected by autoradiography. The size of fragments are expressed in bp.

agar with or without oligomycin. Only a fraction of cells were capable of growing on the drug-containing medium (Table 1). This proportion dropped to a few % after two days growth in the absence of oligomycin. A second subcloning of the remaining resistant clones again gave rise to a high proportion of sensitive clones. It is unlikely that this was due to a slowness of mitotic segregation of mutant mitochondria because the successive clonings continued to give rise to drug sensitive clones at high frequencies.

Although a large majority of the primary resistant clones were unstable, they appear

Figure 3. Location of the or3 mutant DNA on the map of *K. lactis* mt DNA. The small segment amplified in the mutant is shown by a dot to the left of the 15S rRNA gene. The position was deduced from the data of Figure 2 and of a few additional hybridization experiments. The gene map is according to ref. 8 and 25.

to produce, with a relatively high frequency (quantitative determinations were not made), stable secondary clones. When the primary resistant mutants were plated on the drug containing plates for a prolonged period (2–3 weeks), many small colonies grew on the surface of the old large colonies. These subcolonies were, once purified by subcloning, completely stable (see Discussion).

In order to determine the genetic nature of the unstable resistance phenotype, one of the mutants, or3, was crossed with a sensitive wild type strain and subjected to tetrad analysis. Since the cross was made on drug-free medium, a large fraction of the diploids obtained were drug-sensitive and, after meiosis, segregated 0 (resistant): 4 (sensitive) in 16 tetrads, and 1:3 in one tetrad. When oligomycin resistant cells among the diploids were first selected by replica plating on drug-containing medium, and allowed to sporulate in drug-containing medium, all of the sixteen tetrads analyzed showed 4:0 segregation, suggesting a cytoplasmic origin of the mutation.

The unstable oligomycin resistance is associated with transient amplification of a mtDNA segment.

We therefore examined the mtDNA in the or3 mutant grown in the presence of oligomycin. The mtDNA appeared normal in terms of amount and buoyant density in CsCl. However, restriction enzyme digests of the mutant DNA revealed a difference when compared with the wild type DNA (Figure 1). The mutant mtDNA contained an additional DNA fragment which was detected as an intensely staining band, superposed on the normal restriction pattern of mtDNA. The fragment was only observed when the DNA was digested with several particular enzymes (HaeIII, Sau96I, Sau3AI, SacII, SspI, etc.), but not with other enzymes (AvaI, BamHI, EcoRI). The size of the fragment was about 400 base-pairs (bp) in all detected cases. In order to determine whether the fragment originated from the mtDNA, the 400 bp DNA was purified and radiolabelled, then hybridized to the mtDNAs of the wild type and the mutant strains (Figure 2). It hybridized specifically with a short region of mtDNA, localized between map units 7.6 and 8.0 (Figure 3). Therefore, the extra fragment is a segment of mtDNA sequence.

Further restriction analyses suggested that the fragment existed in the form of a large repetitive molecule in which the repeating unit contained a single site for each of the above mentioned particular enzymes (HaeIII etc.), but no sites for others (AvaI etc.). The enzymes which produced the 400 bp fragment were those which cut only once within the above specified region of mtDNA, as predicted from the restriction map.

The existence of such repetitive molecules was demonstrated by partial digestion of the mutant mtDNA. When a partial SspI digest was probed with the radioactive 400 bp DNA, we could detect a polymeric series of sequences which were multiples of about 400 bp. Up to at least octamer size fragments could be detected (Figure 4a).

Therefore, we concluded that the additional DNA in the mutant was a tandemly repeated molecule. The amplified DNA appears to be an independent entity and not an integral part of the mtDNA in the mutant because, if the latter were the case, the restriction patterns of the mtDNA would have shown a readily detectable change with respect to the wild type map when digested with the enzymes which do not cut within the repeating unit. Such changes were not detected. When undigested mutant mtDNA was electrophoresed and hybridized with the radioactive 400 bp probe, we observed a strong smear spanning the molecular weight range of 5–20 kbp. The pattern was the same when the mutant DNA was digested by BglII or HindIII + BamHI (none of which cut within the 400 bp region). In contrast, the digestions of the wild type mtDNA gave a discrete signal whose molecular

Figure 4. Repetitive nature of the *or3* mtDNA. (a) MtDNA from the *or3* mutant and from the wild type was partially digested with *Ssp*I, electrophoresed, and transferred to a nitrocellulose filter. The radioactive probe and the hybridization condition are the same as in Figure 2. (b) The same mtDNAs were completely digested with the indicated enzymes and treated as in (a); the first five lanes are ethidium-stained gels and the last four lanes are autoradiography patterns.

weight was that expected from the map (*Bgl*II 11 kbp, *Hind*III 16 kbp) (Figure 4b).

These experiments led us to conclude that the oligomycin-resistant mutant contains a tandemly amplified segment of mtDNA, and that the amplified molecules, probably of heterogeneous multimer size, are replicating together with the wild type mtDNA within the mutant cells.

Reversion to drug sensitivity is associated with the loss of the amplified DNA.

We examined the mtDNA of one of the spontaneous drug-sensitive revertants (which are produced in large numbers on drug-free plates). We found the normal restriction patterns, with no additional bands (Figure 5). This clone, like two other independently isolated

Figure 5. Restriction patterns of mtDNA from a drug-sensitive revertant of the or3 mutant. MtDNA was isolated from the wild type, the or3 mutant and a revertant of or3. Restriction digests with the indicated enzymes were electrophoresed and visualized by ethidium staining. The revertant lost the additional band of the or3 mutant.

sensitive clones, behaved as a true revertant: when re-plated on a drug containing media, resistant mutants reappeared with a frequency of about 10^{-5} , as in the case of the original strain, and they were all unstable (50 colonies tested).

Nucleotide sequence of the amplified segment of mtDNA.

The amplified segment contained a single Sau3AI site per repeat unit. This 400 bp-long Sau3AI fragment was cloned and sequenced. The corresponding region in the wild type mtDNA was also sequenced. (Figure 6). Comparison of these sequences revealed that the repeating unit had been excised between two short direct repeats, CCCC GCG, present in the wild type mtDNA, 398 bp apart. This short sequence forms part of the many guanine-cytosine (GC) rich clusters that characterize *K. lactis* mtDNA (8). The amplified fragment

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ccttaaatagggataatttagcgagatgttttaacttagcccccgcgcaccccaacgcctacgg 70
                                     ↳ thr2 tRNA
tgggtgagccgcgcggggaatctatatagttaatagtagttatattagctcaattggtagagcgt 140
HaeIII                               ↳ glu tRNA
TCGTTTGTAAATCGAAAGGTTTGGAGTTCGAATCTCTAATAACAGACCTTATCGTCTAATGGTTACGA 210
AsuII
CATCATCTCTTCATGTTGTTAAATATCGGTTCAATTCGGATTAAAGGTTAtatattttataatcccttct 280
c-ccccgcacatgacccctcataaagggcatgctgcggggcctggatatattaccaagtatatccgtggg 350
ATATATTAGAAGTAATATATAATTATAAAATAAATAATGAATATACATATATATTATATATGTACAAATT 420
ACTTGATAATAATATATTATTATATTATAATAATTATTATATATATATTATTATATATAATAACA 490
TAATATTATATTATAAAATATTATTATCTCCGCGAAATTATAAATAATTCGCGGGGATATTCATA 560
SspI
TAAATAATATATAAATTATACATAATTATATTATAATTATATATTTCCCTTTCTCCGCTGGCA 630
TGACCCGACCCCTCATAAAGGGTCGGGTATCCGCGGGGTCATATTTATATAAGGGCccgcgcgctctctc 700
Sau3AI                               Sau96I HaeIII
caccgacttgctcgtgggaatatataatatattatattatatattatatttatatatat 770
atttaaaatataataattcaataaaatgctattta...

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Figure 6. Nucleotide sequence of the mtDNA segment amplified in the or3 mutant. The region indicated by a filled circle in Figure 3 was sequenced. Two tRNA genes (upper case letters), threonyl 2 and glutamyl, are contiguous. Their 5' and 3' ends are indicated by small arrows and dots. A possible transcription start site is underlined. Two septanucleotide direct repeats are boxed. The sequence between these repeats plus one copy of the repeats form the repeating unit of the amplified mtDNA in the mutant or3 (thick upper case letters). Palindromes (not always perfect) are indicated by arrowed underlines. Major restriction sites are specified below each site.

also contained three internal GC clusters which were, like many other clusters, palindromic. In the wild type mtDNA, we found the threonyl 2 (UGU) and glutamyl tRNA genes 27 nucleotides upstream of the region amplified in the mutant. It is interesting to note that the two tRNA gene sequences are contiguous, without a single nucleotide between them. Several nucleotides upstream of the threonyl tRNA gene, there is a possible transcription start signal, ATATAAGTTA (consensus: ATATAAGTA or TTATAAGTA) (9, 10).

Transcription of threonyl and glutamyl tRNA genes.

Mitochondrial mutations conferring oligomycin resistance on *S. cerevisiae* have long been known (11, 12). The resistance has been shown to be due to a mutation in either the ATPase subunit 6 or subunit 9 structural genes. These mutants are stable and respiratory sufficient.

In *K. lactis* mtDNA, the ATPase subunit genes are located far from the region involved in the amplification phenomenon of the or3 mutant. Furthermore, mutations in the ATPase structural genes would result in a classical stable type of resistance. The transient, or reversible, resistance has to be explained by a different mechanism. Since the two contiguous tRNA genes, threonyl and glutamyl, are situated next to the excision-amplification point, we suspected that an anomaly in these tRNAs might be responsible for the resistant phenotype. We therefore examined the transcription and RNA processing of this region. Using probes containing the tRNA genes, we looked for the precursors of threonyl and glutamyl tRNAs. Although the level of tRNA precursors was generally low, two precursor forms containing glutamyl and/or threonyl tRNAs were detectable in the wild type (1600 and 280 bp, Figure 7). In contrast, the mutant RNA contained much reduced amounts of these species and, instead, a 140 bp RNA was apparent, the size of a dimer tRNA. Although the precise structure of these precursors remains to be established, this observation suggests that modified tRNA processing may be involved in the resistance phenotype. Mature forms of these tRNAs were present in normal amounts in the mutant (possibly increased amounts, as judged from shortly exposed autoradiographs). This was expected since the mutant contains the wild type genome, and is growing on a respiratory substrate.

Figure 7. Abnormal transcription product of the threonyl/glutamyl tRNA gene cluster in or3 mutant. MtRNA was extracted from the wild type and the or3 mutant, as described in Materials and Methods. After electrophoresis, RNA was detected by ethidium bromide staining (Left), then transferred to a Hybond sheet and hybridized with a ³²P-labelled probe (Right). The probe was a 494 bp segment of wild type mtDNA including a short fragment of thr2 tRNA gene, glu tRNA gene and most part of the amplified region (AsuII site to Sau3A site, Figure 6); a probe containing only threonyl tRNA and its 5' flanking sequence (a 1100 bp fragment beginning at a Sau3A site and terminating at the HaeIII site in the thr 2 tRNA gene) gave a similar result, showing the presence of the 140 bp signal in the mutant RNA.

DISCUSSION

Basis of the genetic instability.

Unstable mutations often reflect various forms of genome rearrangement. In the case of mitochondrial oligomycin resistance, only stable mutations have been well studied so far. They have been identified in *S.cerevisiae* as point mutations in the mitochondrial ATPase subunit 6 and subunit 9 genes (13, 14). Some nuclear mutations have also been described in an early report (23). Such stable mutations seem to exist also in *K.lactis* which are of either cytoplasmic or nuclear origin (4).

Although a large majority of the primary resistant mutants we isolated were unstable, very stable mutants can be obtained from them after prolonged growth on the drug (Table 1, clone or3*). It is likely that these stable clones correspond to mutations in the ATPase

subunit genes mentioned above. Indeed, the mtDNA of these stable mutants showed normal restriction patterns, with no amplified segments. In the early search for mitochondrial genetic markers in *S.cerevisiae*, only stable mutations had been retained. The existence of unstable clones had been noticed, but they have not been analyzed so far.

In the present work, we tried to understand the basis of this genetic instability. We found that the mutation was cytoplasmic, and that, unexpectedly, it was accompanied by an amplification of a mtDNA segment. This tandemly amplified structure was quite analogous to that of the ρ^- mtDNA. Since the amplified molecule is replicating, the repeating unit should contain a sequence(s) with an *ars* activity, as in the case of ρ^- mutants. It is not known whether the segment contains a unique replication origin of *K. lactis* mtDNA or if there are many such regions endowed with an *ars* activity. It is more likely that the 405 bp region contains a fortuitous weak *ars* sequence which allows the amplified molecules to co-replicate with the wild type genome (which is present in the mutant, in contrast to the stable ρ^- situation), provided that the selective pressure was maintained by the presence of the drug.

Co-replication of ρ^- type repetitive mtDNA with the wild type genome has been known in *S.cerevisiae*. The most illustrative are the cases of the unstable mitochondrial suppressors. These suppressors (15, 16) are associated with an amplification of specific segments excised from the mtDNA. Because of their tandem arrangement, modified versions of mitochondrial genes are created which can bypass their normal regulatory interaction with nuclear gene products. In another case (17), a mutation in the mitochondrial aspartyl tRNA gene was suppressed by an amplification of this gene so that the reduced activity of the modified tRNA was compensated for by an increased production of this molecule. All these unstable suppressors seem to be maintained only under selective pressure on respiratory substrates.

Basis of the oligomycin resistance.

The situation presented here for *K. lactis* may be correlated with the above cases. It is not clear, however, how the oligomycin resistance could be created by the amplification of the 405 bp mtDNA segment. In many well studied cases of stable oligomycin resistance mutations, one could identify, in the ATPase subunits 6 and 9, a 'hot spot' of mutation around a conservative glutamic acid residue (13, 14). This area corresponded to the drug binding site where amino acid substitutions lead to oligomycin resistance. In the present case, if such a modified subunit should be produced, in addition to the normal one, we might suspect the involvement of a post-transcriptional mechanism. Since the processing of tRNA in the mutant seems to be modified concomitantly with the amplification of the downstream sequence, an abnormal processing of the threonyl-glutamyl tRNA precursors might lead to an increased frequency of misacylation, thus producing a modified ATPase subunit. Such a possibility was suggested by the notion that the accuracy of aminoacylation *in vivo* requires proper balance between a tRNA and its cognate aminoacylase (18, 19). Allowing an inaccuracy of protein synthesis may be a device to alleviate the drug effects. The importance, or even necessity, of misacylation in organellar protein synthesis has recently been discussed by Schön et al.(20). We remain at present with the unsolved questions: (i) how amplification of a specific mtDNA segment can modify the processing of threonyl and glutamyl tRNAs, and (ii) how this modified processing is related to the drug resistance.

Whatever the mechanism of resistance, the mitochondrial amplification is a reversible response of yeast to the effect of oligomycin. Once the drug is removed, the cell can recover

the original genome composition. The simultaneous occurrence of normal and modified genomes may be facilitated by the multicopy nature of the mitochondrial DNA and may constitute an advantage for the cell in a changing environment. Many cytoplasmic erythromycin resistance mutations in *K. lactis* were also found to be unstable (unpublished results), suggesting that transient amplification of mtDNA segments may not be rare in drug resistance. The capacity to generate *rho*⁻ type amplifications has been found in various fungal species (21, 22, 23, 24).

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