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Mitochondrial DNA defects in *Saccharomyces cerevisiae* caused by functional interactions between DNA polymerase gamma mutations associated with disease in human

Enrico Baruffini^{1,2}, Iliana Ferrero¹, Françoise Foury²

¹Department of Genetics, Biology of Microorganisms, Anthropology, Evolution, University of Parma, 43100 Parma, Italy

²Institut des Sciences de la Vie, Université Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium

Corresponding author: Françoise Foury

e-mail : foury@fysa.ucl.ac.be

Tel : +32 (0)10474691

Fax : +32 (0)10473872

ABSTRACT

The yeast mitochondrial DNA (mtDNA) replicase Mip1 has been used as a model to generate five mutations equivalent to *POLG* mutations associated with a broad spectrum of diseases in human. All *mip1* mutations, alone or in combination in cis or in trans, increase mtDNA instability as measured by *petite* frequency and Ery^R mutant accumulation. This phenotype is associated with decreased Mip1 levels in mitochondrial extracts and/or decreased polymerase activity. We have demonstrated that (1) in the *mip1*^{G651S} (hG848S) mutant the high mtDNA instability and increased frequency of point Ery^R mutations is associated with low Mip1 levels and polymerase activity; (2) in the *mip1*^{A692T-E900G} (hA889T-hE1143G) mutant, A692T is the major contributor to mtDNA instability by decreasing polymerase activity, and E900G acts synergistically by decreasing Mip1 levels; (3) in the *mip1*^{H734Y}/*mip1*^{G807R} (hH932Y/hG1051R) mutant, H734Y is the most deleterious mutation and acts synergistically with G807R as a result of its dominant character; (4) the *mip1*^{E900G} (h1143G) mutation is not neutral but results in a temperature-sensitive phenotype associated with decreased Mip1 levels, a property explaining its synergistic effect with mutations impairing the polymerase activity. Thus, the human E1143G mutation is not a true polymorphism.

INTRODUCTION

The mitochondrial genome is replicated by a specific DNA polymerase called DNA polymerase gamma (pol g) [1]. The catalytic subunit of pol g is phylogenetically related to the prokaryotic PolA family and its amino acid sequence has been well conserved during evolution from yeast to human. The N-terminal part of the protein contains two domains endowed with dRP-lyase and 3'-5' exonuclease proofreading activities, respectively, and is separated from the C-terminal polymerase domain by a linker region whose length and complexity have increased from yeast to human. While yeast pol g contains a single catalytic polypeptide of ~140 kDa encoded by the *MIP1* gene [2], human pol g is a heterotrimer [3] formed by the 140 kDa catalytic subunit encoded by the *POLG* gene and two identical 55 kDa accessory subunits which increase the activity and processivity of the enzyme.

The first *POLG* mutations associated with an autosomal dominant form of progressive external ophthalmoplegia (adPEO) were reported in 2001 [4]. Six years later more than 80 exonic mutations have been identified. (Human DNA Polymerase Gamma Mutation Database at <http://dir-apps.niehs.nih.gov/polg>). They are associated with a broad spectrum of mitochondrial disorders [5] and [6] including PEO, myopathy, Parkinsonism, premature menopause, psychological disorders, ataxia, encephalopathy, and the fatal Alpers syndrome. The affected tissues are characterized by multiple deletions or depletion of mtDNA. *POLG* mutations are often pathogenic as compound heterozygous. However, the mechanisms underlying symptom variability are not understood. Moreover, as several *POLG* mutations are often simultaneously present in the patients, it is difficult to distinguish between single nucleotide polymorphisms (SNPs) and pathogenic mutations. For instance, the E1143G substitution identified in 2-3 % of the Northern population and met in healthy homozygous

individuals has been considered as a SNP. However, it is also very often associated with pathogenic mutations in cis or in trans [7], [8], [9], [10] and [11].

The *MIP1* gene has previously been used in *Saccharomyces cerevisiae* as a model to study pathogenic *POLG* mutations [12] and [13]. Moreover, *S. cerevisiae* appears as an appropriate organism to search for genes and mechanisms rescuing the defects generated by deleterious *POLG* mutations [13] and [14]. In the present study five human *POLG* mutations mapping to the polymerase domain and associated with a large range of clinical symptoms, from mild PEO to severe neurological manifestations and Alpers syndrome, were introduced at the homologous positions in the *MIP1* gene, and compound heterozygous yeast strains as found in human patients were constructed. The effects of the *mip1* mutations on mtDNA stability and point mtDNA mutation rate, dominance/recessivity of the phenotypes and DNA polymerase activity were analysed. We found a good correlation between mtDNA maintenance in yeast and disease severity in human.

MATERIALS AND METHODS

Yeast strains and media

The *Saccharomyces cerevisiae* strains used in this work are W303-1B (*Mata ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100*), DWM-5A (*Mata ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR*) and DWM-1A, its isogenic *Mata* counterpart. DWM-1A and DWM-5A are meiotic segregants derived from W303 [13].

The diploid DMM was obtained by crossing DWM-5A which harbours plasmid pFL38*MIP1* (see below) and is *rho*⁺ with DWM-1A which harbours plasmid pFL39 (see below) and is *rho*⁰. A diploid that has lost pFL39 and retained pFL38*MIP1* was then selected.

YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (Difco). YPA medium was YP medium supplemented with 40 mg/l adenine. Synthetic defined (SD) medium contained 6.7 g/l yeast nitrogen base without amino acids (Difco) supplemented with appropriate amino acids and bases. Synthetic complete (SC) medium contained 6.7 g/l yeast nitrogen base without amino acids (Difco) and supplemented with 1g/l of drop-out powder [15] containing all amino acids except those required for plasmid maintenance. Carbon substrates were added at the indicated concentration. YPAEG-ery contained 1% Bacto-yeast extract, 2% Bacto-peptone (Difco), 40 mg/l adenine, 3% ethanol, 3% glycerol, 3 g/l erythromycin (SIGMA) and 25 mM potassium phosphate buffer at pH 6.5.

Construction of yeast *mip1* strains

The *mip1* mutations were obtained by site-directed mutagenesis of a *MIP1* gene fragment, using the overlap extension technique [16] and after appropriate restrictions the *MIP1* and *mip1* alleles were subcloned in the *SacI* and *SalI* sites of the centromeric pFL38 (*URA3* marker) or pFL39 (*TRP1* marker) plasmids [17]. The pFL39 plasmid-borne *MIP1* and *mip1*

genes were transformed in the DWM-5A strain deleted for the *MIP1* gene and harbouring the pFL38 plasmid-borne *MIP1* gene to allow mtDNA maintenance. The plasmids were named pFL39*MIP1* (or *mip1* followed by the name of the mutation) and pFL38*MIP1* (or *mip1*). In a second step, the pFL39-borne *MIP1* or *mip1* alleles were selected through plasmid shuffling using 5-fluoroorotic acid as previously described [13]. Heteroallelic haploid strains were obtained by transformation of the pFL39*MIP1* or pFL39*mip1* plasmid-bearing strains with pFL38-based plasmids.

The pGAL*mip1* plasmids containing the desired mutation were constructed by replacing the *NotI-NgoMIV* fragment of the wild-type *MIP1* gene borne by the pGAL*MIP1* plasmid [18] by the corresponding mutated fragment. Strain W303-1B was transformed with pGALZ3 (the empty plasmid), wild-type pGAL*MIP1* and pGAL*mip1* mutant plasmids. The presence of the *mip1* mutation was verified by DNA sequencing.

Nomenclature

DWM-5A strains harbouring *mip1* mutations were named as follows. The *mip1* symbol was followed by the residue number preceded by the one-letter-code symbol of the wild-type amino acid followed by the symbol of the mutant residue. Thus *mip1*^{G651S} means *mip1* mutant harbouring a mutation from glycine to serine at position 651 of the amino acid sequence. Heteroallelic strains harbouring pFL38 plasmid-borne *MIP1* and pFL39 plasmid-borne *MIP1* (or *mip1*) alleles, or empty pFL39 plasmid were designated as *MIP1*(or *mip1*)/*MIP1*, or *MIP1*/pFL39 strains. Strain *mip1*^{H734Y}/*mip1*^{G807R} was obtained by transforming the DWM-5A strain harbouring the pFL39 plasmid-borne G807 mutation with the pFL38 plasmid bearing the H734Y mutation. Strain *mip1*^{G651S}/*mip1*^{E900G} was obtained by the same procedure.

Determination of *petite* frequency and mtDNA retention

Strains containing *MIP1* or *mip1* alleles were grown on solid SC medium supplemented with 2% ethanol at 28°C to counterselect the *petite* cells that could be present in the population. After approximately 60 h, strains were replica-plated on SC medium supplemented with 2% glucose and grown at 28°C or 36°C. After 24 h, strains were replica-plated on SC medium supplemented with 2% glucose and grown at 28°C or 36°C. After 24 h cells were diluted in water and plated on SC agar plates supplemented with 2% ethanol and 0.3% glucose at a dilution giving approximately 200 cells per plate. *Petite* frequency was defined as the percentage of colonies showing the *petite* phenotype after a 5 day-incubation at 28°C. For each strain at least 4000 clones were analysed. When 100% *petites* were obtained, 10^4 and 10^5 cells were directly plated on YP plates supplemented with 3% glycerol to confirm that the cell population was only composed of *petites* and did not grow on glycerol.

To determine whether *petites* had retained mtDNA fragments, the clones in the DWM-5A background were crossed with *mit* mutants, M9-94-4B (*Mata ade1 cox2*), M7-40-5B (*Mata ade1 cob*), M17-162-4B (*Mata ade1 cob*) and M9-3-5B (*Mata ade1 cox3*) [19] as previously described [13]. *Mit* mutations are mtDNA point mutations conferring respiratory deficiency and loss of growth on a respiratory carbon source [20]. Briefly, for each haploid *mip1* mutant, at least 200 independent *petite* clones were crossed with the *mit* mutants of opposite mating type on YP plates supplemented with 2% glucose and replica-plated on YP plates supplemented with 3% glycerol to select *rho*⁺ diploid strains. For the *mip1*^{G651S} mutant, 200 independent clones were crossed with *mit* mutants immediately after isolation of the mutant strain.

Frequency of erythromycin resistant (Ery^R) mutants

Two independent series of 10 independent colonies grown on YP plates supplemented with 3% glycerol were inoculated in 2.5-ml cultures of YPA supplemented with 3% glycerol. After

48 h at 28°C, 1.5 ml of each culture, corresponding to $6-8 \times 10^7$ cells, were plated on YPAEG-ery medium and incubated at 28°C for 9 days. A small aliquot of each culture was spread for single colonies on YP plates supplemented with 3% glycerol to determine the number of *rho*⁺ cells initially present in the cultures. The mutation frequency was calculated as the number of Ery^R colonies per 10^8 *rho*⁺ cells. For heteroallelic strains containing both pFL39- and pFL38-derived plasmids, the same test was carried out, except that colonies were grown on SD plates supplemented with 2% glucose and then inoculated in 2.5-ml cultures of SC medium supplemented with 2% ethanol to avoid loss of one plasmid.

Southern hybridisation

Cells pre-grown on SC medium supplemented with 2% ethanol (except for mutant *mip1*^{G651S} which was supplemented with 2% glucose) were inoculated in 10 ml of SC medium supplemented with 2% glucose and grown overnight. Total DNA was extracted by standard procedures, digested with *Eco*RI, and subjected to electrophoresis in an 0.8% agarose gel. The *TRP1* alleles harboured by the pFL39*MIP1* plasmid and the genome are contained within DNA fragments of 4.8 and 1.45 kb, respectively. Hybridisation was performed by standard procedures with the *TRP1* gene as a probe using [α -³²P]dCTP and the kit 'Rediprime II DNA Labeling System' (GE Healthcare). The *TRP1* probe was obtained by *Bgl*III digestion of the pFL39 plasmid. The signals were quantified after a 15-min exposure with a PhosphorImager (Molecular Dynamics).

DNA polymerase assay

Mitochondrial extracts were obtained from strain W303-1B overexpressing the *MIP1* (*mip1*) alleles under the control of the *GAL1* promoter in a 2% galactose-containing SC medium for 15 h at 30°C as previously reported [18]. The W303-1B strain harbouring the empty GALZ3

plasmid was taken as the negative control. The gap-filling activity assay was carried out in a reaction mixture containing 20 mM Tris-HCl pH 8.0, 5 mM dithiothreitol, 50 mM MgCl₂, 25 μM each dATP, dCTP, dGTP, 6 μM [³H]-dTTP, 150 μg/ml calf thymus activated DNA and the mitochondrial extract. Samples were taken after 6 min of incubation at 30°C. The activity was expressed in nmol dTTP incorporated into trichloroacetic precipitable material per h and per mg protein present in the extract or was reported to an arbitrary amount of Mip1 (100 for the wild-type).

RESULTS

Choice of *POLG* mutations and construction of *mip1* mutants

Positions of the mutations studied in this work are shown in Fig. 1A.

The recessive **G848S** mutation which affects a conserved glycine residue upstream of motif polA (Fig.1B) in the polymerase domain is involved in diseases of variable severity, from PEO to the Alpers syndrome, according to the associated mutations in trans [9], [21], [22], [23], [24], [25] and [26] (Table 1). The **A889T** mutation affects a conserved residue immediately upstream of motif polA (Fig. 1B). This mutation associated in cis with the SNP **E1143G** (Fig. 1B) has been reported as dominant in two brothers with PEO and severe neurological manifestations [8] (Table 1). Their mother and two grand-uncles, obligate mutation carriers, showed only tremors, suggesting that genetic or environmental factors modulates the severity of the phenotype. The A889T mutation has also been reported associated in trans with the R579W mutation in a brother and a sister with PEO, sensory ataxia, areflexia, and gastrointestinal dysmotility [27]. Since in this case parents were healthy A889T seems, when alone, to be a recessive mutation. However, since one *POLG* mutation in different families and individuals may cause different kinds of disorders it cannot be excluded that A889T alone is dominant. Nevertheless, the most likely hypothesis is that E1143G modifies the pathogenic effect of A889T.

The **H932Y** mutation affects a strictly conserved residue slightly upstream of motif polB (Fig. 1B). The **G1051R** mutation affects a residue located between motifs polB and polC within a region poorly conserved in prokaryotic DNA polymerases (Fig. 1B). H932Y/G1051R compound heterozygous patients developed a complex syndrome including PEO, polyneuropathy, ataxia, hearing loss and psychiatric disorders [28] (Table 1).

The equivalent *mip1* mutations G651S (hG848S), A692T (hA889T), H734Y (hH932Y), G807R (hG1051R) and E900G (hE1143G) obtained by site-directed mutagenesis (Fig. 1A) were introduced in the *MIP1* gene borne by a low copy centromeric vector and the *mip1* alleles were cloned in a yeast strain whose chromosomal *MIP1* gene was deleted. The *MIP1* allele used in this work is found in several commonly used laboratory strains including the S288c reference strain and W303. It contains an alanine at position 661. However, T661 is a strictly conserved residue from fungi to human, and all natural *S. cerevisiae* strains contain a threonine at this position. We have recently shown that the T661A substitution is a mutation increasing mtDNA instability in a temperature-dependent manner [29]. Here, the choice of the A661 context is based on two previous reports on the effect of pathogenic *POLG* mutations which were using the Ala661-containing *MIP1* gene as a model [12] and [13]. In the last section we present a comparison of the mutant phenotypes obtained in the A661 and T661 contexts.

Southern blotting experiments were performed to estimate the average copy number of pFL39 plasmid-borne *MIP1* (*mip1*) gene. Even though it was not excluded that mutants *mip1*^{G651S} and *mip1*^{H734Y} contained on average slightly more than one copy per cell, chromosomal and plasmid DNAs were grossly in stoichiometric amounts in all mutants (Fig. 2), indicating that copy number of the *mip1* alleles was not influenced by their presence on a centromeric vector.

Effect of *mip1* mutations on oxidative growth phenotype

The analysis of cellular growth on an obligatory respiratory carbon source such as ethanol or glycerol is a simple method to test mitochondrial function. All mutants except *mip1*^{G651S} were able to grow on ethanol at 28°C (Fig. 3A). Whereas mutants *mip1*^{G807R} and *mip1*^{E900G} were growing like wild-type, cellular growth of mutants *mip1*^{A692T} and *mip1*^{H734Y}, and to a greater

extent that of mutant *mip1*^{A692T-E900G}, was impaired. In all mutants the growth defect was more pronounced at 36°C (Fig. 3B).

Effect of single *mip1* mutations on *petite* accumulation

The patients harbouring the mutations studied here present multiple deletions or depletion of mtDNA. While in human cells deleted and intact mtDNA molecules are coexisting, *S. cerevisiae* has the unique property to produce cells with homoplasmic deleted mtDNA molecules, which are referred to as *petites* or *rho*⁻ mutants [30]. These mutants do not grow on respiratory carbon sources but they can grow on glucose thanks to the energy produced by fermentation. In all *mip1* mutants the frequency of *petites* was increased at 28°C, being quite low in *mip1*^{E900G} (6%), moderate in *mip1*^{G807R} (25%) and high in *mip1*^{A692T} and *mip1*^{H734Y} (80 and 99%, respectively) (Table 2). In all strains *petite* frequency was increased at 36°C. The temperature-sensitive trait was specially marked for the G807R and E900G mutations, which yielded 99% and 92% *petites*, respectively (Table 2). No *rho*⁺ colony was obtained for mutant *mip1*^{G651S}, suggesting that mtDNA maintenance was incompatible with the G651S mutation. *Petite* cells either contain amplified mtDNA fragments mapping to various positions of the mtDNA molecule, and are referred here to as *rho*⁻, or are completely devoid of mtDNA and referred to as *rho*⁰. To distinguish between *rho*⁻ and *rho*⁰ clones we used a genetic test based on the capacity of the mtDNA fragments retained by *petite* clones to rescue *mit*⁻ mutations by homologous recombination [31]. The *mit*⁻ mutations were selected in the *cox2*, *cox3* and *cob* genes, because these correspond to the most frequently retained loci in *rho*⁻ mutants [32]. Absence of restoration of these *mit*⁻ mutants to wild-type by a large number of *petite* clones provides a good evidence that the *mip1* mutation leads to total loss of mtDNA. Our data show that there was a good correlation between *petite* frequency and loss of mtDNA (Table 2). In

particular, they strongly suggest that the *mip1*^{G651S} mutant is not able to maintain a fragment of mtDNA, implying a severe defect in mtDNA replication.

Effect of single *mip1* mutations on Ery^R mutant frequency

The fidelity of mtDNA replication was estimated by the frequency of erythromycin (Ery^R) mutants. Ery^R mutations are caused by mutations in the mtDNA 21S rRNA gene (nucleotides 59965-59967 on the mtDNA map in SGD database <http://www.yeastgenome.org/>) [33]. All types of substitutions can be obtained. An increased frequency of Ery^R mutants was observed in all tested *mip1* mutants, but there was no relationship between Ery^R and *petite* mutant frequencies (Table 2).

Dominance/recessivity of *mip1* mutations

Dominance/recessivity of each *mip1* mutation was determined in diploids in which both chromosomal *MIP1* genes were deleted and replaced by a pFL38-borne wild-type *MIP1* gene and a pFL39-borne wild-type *MIP1* or mutant *mip1* gene (Table 3). A diploid strain containing two copies of the *MIP1* gene produced less than 1% *petites* at 28°C. When one *MIP1* copy was replaced by the empty plasmid (pFL39), the *petite* frequency was two-fold higher at 28°C and further increased at 36°C. *Petite* accumulation in the presence of a single copy of *MIP1* is a sign of haploinsufficiency suggesting that Mip1 is a limiting factor in mtDNA replication.

At 28°C, the percentage of *petites* was similar in the *MIP1/mip1*^{E900G} and *MIP1/MIP1* strains, indicating that the *mip1*^{E900G} allele is functional. All other *MIP1/mip1* strains showed haploinsufficiency, to various extents, suggesting that the *mip1* alleles in diploids are poorly functional. In the *MIP1/mip1*^{H734Y} strain *petite* accumulation was significantly higher (7.4%) than in a strain bearing a single copy of wild-type *MIP1* (2.1%), indicating that the *mip1*^{H734Y}

allele is dominant (Table 3). In all strains Ery^R mutant accumulation was recessive (data not shown).

Functional interactions of *mip1* mutations in cis or in trans

In order to simulate human situations, mutants carrying several mutations in cis or in trans were constructed both in diploid (Table 3) and haploid states (data not shown). In the latter context, the frequency of *rho*⁰ clones could be determined in crosses with *mit*⁻ mutants. The presence of the E900G mutation in cis with the A692T mutation exacerbated the mtDNA defects produced by the A692T mutation, but did not further increase the frequency of Ery^R mutants (Table 2). No difference was observed between the *MIP1/mip1*^{A692T} and *MIP1/mip1*^{A692T-E900G} diploid strains (Table 3), leading to the conclusion that the presence of the E900G mutation in cis does not result in a dominant phenotype, as suggested in human for the E1143G substitution associated in cis with the A889T mutation.

When two plasmid-borne *MIP1* (or *mip1*) genes were introduced into a haploid instead of a diploid strain, *petite* frequency was quite similar (compare *MIP1/MIP1*, *mip1/pFL39*, *MIP1/mip1*^{G807R}, *MIP1/mip1*^{H734Y} in Tables 3 and 4). Strains *mip1*^{H734Y}/*mip1*^{H734Y} and *mip1*^{G807R}/*mip1*^{G807R} produced 97.8% and 8.7% *petites*, respectively (Table 4). *Petite* accumulation in the heteroallelic haploid *mip1*^{H734Y}/*mip1*^{G807R} strain reached 70.7% (versus 83.9 in a diploid strain in Table 3), with a high frequency of cells devoid of mtDNA (Table 4), indicating that the mild G807R mutation was unable to compensate for the deleterious effects of the H734Y mutation. In agreement with these data the *mip1*^{H734Y}/*mip1*^{G807R} strain grew on ethanol-containing medium slightly better than the *mip1*^{H734Y}/*mip1*^{H734Y} strain but more slowly than the *MIP1/pFL39* and *MIP1/mip1*^{G807R} strains (Fig. 2C). The dominant trait of the H734Y mutation was thus confirmed, providing support to the view that the human equivalent H932Y is dominant. The frequency of Ery^R mutants was hardly decreased in the

compound heterozygous strain compared to the *mip1*^{G807R}/*mip1*^{G807R} strain (Table 4), implying that the contribution of Mip1^{H734Y} polymerase to the fidelity of mtDNA replication was small.

In haploid or diploid *mip1*^{E900G}/*mip1*^{G651S} strains *petite* frequency reached 6-6.9%, a percentage higher than in the strain homozygous for the E900G mutation (~2%) and similar to that of a strain bearing a single copy of the E900G mutation, supporting further the hypothesis that *mip1*^{G651S} behaves like a null allele.

Levels and gap-filling activity of wild-type and mutant Mip1p

POLG mutations in the polymerase domain can be ranged into two broad classes. a) Mutations altering protein conformation, as a result of an intrinsic folding defect or an alteration of protein interactions with the DNA substrate. b) Mutations impairing polymerase activity, and altering, or not, the protein structure.

Mip1 is naturally poorly expressed and gives a very faint signal in Western blots (Fig. 4A). Therefore, soluble mitochondrial extracts were prepared from cells overexpressing the *MIP1* gene from the strong inducible *GALI* promoter. We first verified that *MIP1* gene expression was similar in all strains (data not shown). However, in all mutants, the amount of Mip1 in the mitochondrial extracts was lower than in wild-type (Fig. 4B and C, and Table 5), but to various extents. Whereas the amounts of Mip1^{A692T} were quite comparable to that of the wild-type, those of Mip1^{G651S}, Mip1^{G807R} and Mip1^{A692T-E900G} were very low (<10% of wild-type Mip1), and those of Mip1^{H734Y} and Mip1^{E900G} were substantially reduced (Fig. 4B and C and Table 5). By comparison, the extracts of a strain expressing the chromosomal *MIP1* gene contained 1.5-2% of the amount present in the overproducing wild-type strain (Table 5).

Gap-filling activity measures the capacity of incorporating nucleotides into short gaps of excess duplex DNA and is thus independent of the processivity of the enzyme. A decrease in

this activity reflects mainly a defect in catalysis or a decrease in K_m (dNTP). In all strains data were normalized to a same amount of Mip1 protein in the assay. Gap-filling activities of Mip1^{E900G} and Mip1^{G807R} were not modified (Mip1^{E900G}) or close (Mip1^{G807R}) to that of wild-type (Table 5). Together with the observation that in these mutants Mip1 levels are decreased, these data suggest that the mtDNA replication defects of these mutants mainly result from limiting amounts of Mip1. In contrast, the activity of Mip1^{A692T} reached only 30% of that of the wild-type. Since Mip1^{A692T} levels were close to that of wild-type, it could be concluded that the mtDNA replication defects produced by the A692T mutation are mainly caused by an intrinsic defect in the polymerase activity of the enzyme. The gap-filling activity of Mip1^{A692T-E900G} was similar to that of Mip1^{A692T} (Table 5). Since the major effect of the E900G mutation in the presence of the A692T mutation was a dramatic decrease of Mip1 levels in the extracts (Fig. 4 and Table 5), the low mtDNA replication capacity of the *mip1*^{A692T-E900G} mutant was better explained by the cumulative effects of low polymerase activity caused by A692T and low protein levels caused by E900G. The Mip1^{G651S}, and to a lesser extent, Mip1^{H734Y} polymerases combined decreased gap-filling activity and lower expression.

Comparison of the *MIP1*^{T661} and *MIP1*^{A661} alleles

As mentioned earlier in this paper the T661A substitution present in the *MIP1* gene used in this work decreases mtDNA stability of ‘wild-type’ laboratory strains [29]. To determine the influence of the T661A substitution, the mutant phenotypes were compared in the A661 and T661 contexts (Tables 2 and 6). Although mutability and temperature sensitivity were remarkably more pronounced in the presence of A661, there was no fundamental discrepancy in the two genetic contexts. (1) G651S and H734Y strongly increased *petite* frequency; (2) E900G increased *petite* frequency approximately by two-fold; (3) A692T and G807R

increased *petite* frequency at an intermediate level; (4) E900G behaved as a phenotypic modifier of A692T in cis; (5) in all mutants the frequency of point Ery^R mutants was increased. (6) The dominant or recessive character of the mutations, and more specifically H734Y dominance, were not influenced by the A661 or T661 context (data not shown). However, the haploinsufficiency observed in the presence of a single *MIP1* copy instead of two had disappeared in the T661 context (data not shown). Moreover, whereas in *mip1*^{G651S} mutant mtDNA was not maintained in the A661 context, preventing further analysis of point mutant accumulation, in the T661 context almost half of the cell population was still *rho*⁺ at 28°C and showed an increased rate of Ery^R point mutations (Table 6).

DISCUSSION

The question addressed in this work was: Is *Saccharomyces cerevisiae* a good model to determine the effect of each individual mutation in patients harbouring several *POLG* mutations in cis or as compound heterozygous? Indeed, except in the case of dominant PEO, the patients often carry more than one *POLG* mutation in cis or in trans, and thus the specific contribution of each mutation to the disease cannot easily be established.

In this work we have shown that all the single substitution *mip1* mutations studied here cause mtDNA defects, increasing the frequency of deletions and in some cases the frequency of point mutations. We have found a good correlation between decreased cellular oxidative growth, *petite* frequency and loss of mtDNA. In contrast, there is no clear relationship between *petite* and point mutation frequencies. Although the two *MIP1* alleles used in our study, T661 and A661, confer very different levels in mtDNA instability, the classification of these mutations is similar. From the highest to lowest severity the ranking is G651S (hG848S) and H734Y (hH932Y) > A692T (hA889T) > G807R (hG1051R) > E900G (hE1143G).

The extreme severity of the G651S mutation fits with the observation that patients compound heterozygous for G848S and A467T, or G848S and W748S-E1143G mutations present with the fatal Alpers syndrome [22], [23] and [24].

Whereas the relative contribution of mutations H932Y and G1051R in trans could not be established in the patients, comparison of the severity of the yeast H734Y and G807R mutations strongly suggests that the major contributor to the disease is H932Y, and moreover, our data show a strong synergistic deleterious effect of the two mutations associated in trans. In addition, the family history was showing that individuals heterozygous for the H932Y mutation were developing mild symptoms or were asymptomatic, but conclusions were obscured by the presence of migraines and psychiatric disorders of unknown origin in several

members of the family [28]. Our data in yeast have demonstrated that both in the A661 and T661 contexts, H734Y is dominant, suggesting that heterozygosity for H932Y has potential to cause symptoms in human.

Because in the patients A889T, the equivalent of yeast A692T, was associated either with R579W in trans [27] or with E1143G in cis (the equivalent of yeast E900G), it was not possible to determine the individual contribution of A889T to the disease. Our data in yeast show that, when alone, A692T increases mtDNA instability significantly and E900G has a very mild phenotype. Therefore, it can be concluded that A889T significantly contributes to the disease. However, the association of E900G and A692T in cis has a strong deleterious synergistic effect resulting probably from the combination of a folding defect produced by E900G which decreases pol g levels and an intrinsic polymerisation defect produced by A692T. Therefore, it is possible that in humans symptoms are worsened by E1143G. Since in the carrier family the A692T-E900G mutations were dominant, though to different severity levels, it was concluded that E900G could be responsible for the dominant character. Our data show that the very weak dominant phenotype detected in the *mip1*^{A692T} mutant is not further increased in the *mip1*^{A692T-E900G} mutant, demonstrating that E900G does not confer a dominant phenotype and suggesting that the strong dominant phenotype observed in some members of the family [8] was not caused by E1143G but more likely by other factors interacting with *POLG*.

As discussed above our data show that E900G is not a silent mutation. When alone, it confers a strong temperature-sensitive phenotype in the A661 context, and even in the T661 context, a 5-fold increase in *petite* frequency is observed at 36°C. This temperature-sensitive trait associated with the observation that Mip1 levels are decreased in mitochondrial extracts suggests that the E900G mutation acts on Mip1 structure. Thus, the deleterious synergistic effect of E900G mutation would be better explained by the combination of decreased amounts

of protein caused by E900G and decreased polymerase activity caused by A692T. In contrast with these data, it has been reported that when associated with the W748S mutation in cis as found in patients, E1143G partially rescues the deleterious defects of the W748S mutation in vitro by improving the catalytic activity and the affinity for DNA of the mutant protein [34]. As this is the case in yeast for E900G associated with A692T, the E1143G substitution increases instability of W748S-E1143G pol g, and thus its unfolding propensity at body temperature. However, we do not think that these data, obtained in yeast and human for different mutations, are necessary contradictory. They rather suggest that the effect of E1143G, by modifying protein structure, may depend on the second mutation it is combined with. The effect of the combination of the W748S and E1143G equivalents in yeast could not be studied since Trp748 is not conserved in Mip1.

A matter of debate is whether accumulation of point mutations in mtDNA contributes to the disease. It has been postulated that point mutations near direct repeats are an early event in PEO which contributes to the formation of deletions [35]. On the other hand, muscle sample analysis of PEO patients with enhanced levels of point mutations and deletions [36] revealed that in young patients deletions were observed before any point mutation accumulation (point mutations occurring in an age-dependent manner). In addition, point mutation overload was specifically observed in the control region of mtDNA, whereas deletion break-points were mainly produced close to homopolymeric runs, suggesting that replication stalling was the primary cause of deletions [36]. Moreover, no point mutations were found in 136 single COX-negative fibres from biopsies of PEO patients harbouring multiple deletions [26]. It was concluded that clonal expansion of mtDNA deletions accounts sole for the pathogenesis of mitochondrial diseases. The frequency of point Ery^R mutations is increased in all *mip1* mutants, suggesting that fidelity of mtDNA replication is decreased or mutation fixation is increased. However, each mutant behaves differently, with no correlation between the

frequency of point mutations and deletions. The *mip1*^{A692T} mutant produces a greater number of *petites* than the *mip1*^{G807R} mutant, but a smaller number of Ery^R mutations. The percentage of *petites* is substantially increased in the *mip1*^{A692T-E900G} mutant, compared to the single substitution *mip1*^{A692T} mutant, but the frequency of Ery^R mutants is unchanged. The *mip1*^{H734Y} allele is dominant with regards to *petite* frequency, but it is recessive for Ery^R mutant frequency. This suggests that mtDNA instability is caused in general by different mechanisms from those producing single point mutation, although it can not be excluded that increased point mutability could be involved for some *POLG* mutations. For example, the G651S mutation in the T661 context leads to the highest accumulation of *rho*⁻ clones and point mutations. A better understanding of the role of the mutated pol g residue and mechanisms leading to mtDNA instability will allow to establish the relationship between point mutations and deletions in the different mutants.

Finally, we would like to discuss reasons for using A661 or T661 alleles. The present work has been centred on the A661 allele in order to facilitate comparisons with two previous works on *POLG* mutations [12] and [13], and also because the reference *S. cerevisiae* strain used for large scale functional analyses contains Ala661. In all mutants, except *mip1*^{H734Y}, mtDNA defects were dramatically increased in the A661 background. This was particularly marked for the G651S and E900G mutations. When borne by the A661 allele the G651S mutation results in a null *rho*⁰ phenotype whereas in the T661 context it allows mtDNA maintenance in almost half of the cells at the permissive temperature. Similarly, the temperature-sensitive properties of the E900G mutation in the A661 context are remarkably less pronounced in the T661 context. Thus, although the A661 allele may introduce bias in interpretation of the data, it can also emphasize subtle phenotypes and facilitate a systematic search for phenotypic suppressors.

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LEGEND TO FIGURES

Figure 1. Position and conservation of mutated amino acids associated with mitochondrial diseases in several DNA polymerases gamma and *E. coli* DNA polymerase I. A. Linear representation of human pol g and Mip1 polymerases. I, II and III refer to the ExoI, ExoII, and ExoIII conserved motifs of the 3'-5' exonuclease domain, and A, B and C refer to the polA, polB and polC conserved motifs of the polymerase domain. B. Multiple amino acid alignment of regions encompassing the mutated residues. Numbers refer to the position of the residues in the amino acid sequence. Mutated residues are in bold characters and underlined in *H. sapiens* and *S. cerevisiae*. Alignment of DNA was carried out by using CLUSTAL W program [37].

Figure 2. Estimation of the copy number of pFL39-borne *MIP1* (*mip1*) genes in wild-type and mutants. The *MIP1* (*mip1*) genes were borne by the pFL39 plasmid. The standard deviation of the ratio of the plasmid to genomic DNA was in the range of 0.1-0.3. Southern blotting was performed as described in Materials and Methods.

Figure 3. Oxidative growth phenotype of wild-type, *mip1* and heteroallelic strains. Cell growth was tested on SC medium supplemented with 2% ethanol at 28°C, and on YP medium supplemented with 2% ethanol at 36°C (cells were unable to grow on ethanol supplemented SC medium at 36°C). For each strain, 10^5 , 10^4 , 10^3 and 10^2 cells were spotted. Pictures were taken after 4 days. A. *mip1* mutants at 28°C. B. *mip1* mutants at 36°C. C. Heteroallelic haploid strains at 28°C.

Figure 4. Estimation of wild-type and mutant Mip1 levels in mitochondrial extracts. The extracts prepared as reported in Materials and Methods were subjected to SDS-PAGE in a 7% polyacrylamide gel and Mip1 was detected by Western blotting using a polyclonal antibody raised against Mip1. For each strain, 30 μ l of extract in the solubilisation buffer were loaded onto the gel and the intensity of the signal was compared to that obtained by increasing amounts of the standard wild-type Mip1 expressed under the control of the *GALI* promoter. The wild-type mitochondrial extract contained 2 mg/ml protein. Western blots A, B and C were run independently. A. Mip1 was expressed from the chromosomal *MIP1* gene in cells containing the empty *GALZ3* plasmid and its level was estimated by using a standard Mip1 curve from 0.25 to 1 μ l of extract. B and C. The levels of Mip1^{A692T-E900G}, Mip1^{G807R}, Mip1^{G551S}, Mip1^{H734Y} and Mip1, Mip1^{E900G} and Mip1^{A692T} expressed under the control of the *GALI* promoter were estimated by using a standard curve from 1 to 4 μ l (B) or 1 to 30 μ l (C).

Table 1. Relevant pathologies, human *POLG* mutations and yeast *mip1* equivalents studied in this work

Human <i>POLG</i> mutations		Equivalent <i>mip1</i> mutations		Pathology
Allele 1 ^a	Allele 2 ^a	Allele 1	Allele 2	
G848S	T251I-P587L	G651S	NC ^b	PEO [25] and [26]
G848S	A467T	G651S	NC	Alpers [9] [21] [23] and [24]
G848S	W748S-E1143G	G651S	NC-E900G	Alpers [22] and [23]
A889T-E1143G	Wt ^c	A692T-E900G	Wt	Tremor or adPEO/Neuropathy [8]
A889T	R579W	A692T	NC	PEO/Neuropathy [27]
H932Y	G1051R	H734Y	G807R	PEO/Neuropathy [28]

^aAlleles 1 and 2 are borne in trans by different chromosomes

^bNC, not conserved

^cWt, wild-type

Table 2. *Petite* and Ery^R mutant frequency in *mip1* mutants

Haploid strain ^a	28°C		36°C		Ery ^R mutant frequency ^d	
	% <i>petites</i> ^b	% <i>rho</i> ^{0c}	% <i>petites</i>	% <i>rho</i> ⁰	Ery ^R (x 10 ⁸)	Fold increase
<i>MIP1</i>	2.7 ± 0.3	13	21.0 ± 2.4	37	6.0 ± 0.4	1.0
<i>mip1</i> ^{G651S}	100 ± 0	100	-	-	ND ^e	-
<i>mip1</i> ^{A692T}	80.3 ± 1.7	86	99.7 ± 0.1	100	22.2 ± 2.7	3.7
<i>mip1</i> ^{H734Y}	99.7 ± 0.1	94	100 ± 0	100	ND	-
<i>mip1</i> ^{G807R}	25.5 ± 2.8	55	99.2 ± 0.2	99	61.6 ± 6.1	10.2
<i>mip1</i> ^{E900G}	6.0 ± 0.6	33	91.7 ± 3.9	88	11.6 ± 1.3	1.9
<i>mip1</i> ^{A692T-E900G}	96.6 ± 1.5	96	100 ± 0	100	20.4 ± 1.3	3.4

^aThe DWM-5A strain contains the *MIP1* (or *mip1*) gene borne by the pFL39 plasmid.

^bIn this work ‘*petites*’ designates both *rho*⁻ clones with mtDNA deletions and *rho*⁰ clones. For each *MIP1* (*mip1*) strain the data are averages obtained from three independent experiments and two independent transformants.

^cAlthough not formally demonstrated a *petite* mutant which does not restore any of the four *mit*- tester strains is referred to as *rho*⁰. *Rho*⁰ frequencies are calculated as the ratio of the number of *rho*⁰ to the number of *petite* cells.

^dThe frequency of Ery^R mutants was calculated as the ratio of the number of Ery^R mutants to the number of *rho*⁺ cells plated on the Petri dish. The ‘fold increase’ was calculated by normalization of the wild-type *MIP1* strain relative frequency to 1.

^eND, not determined.

Table 3. *Petite* mutant frequency in heteroallelic diploid strains

Diploid strains	% <i>petites</i> at 28°C
<i>MIP1/MIP1</i>	0.8 ± 0.1
<i>MIP1/pFL39</i>	2.1 ± 0.1
<i>MIP1/mip1^{G651S}</i>	1.8 ± 0.2
<i>MIP1/mip1^{A692T}</i>	2.6 ± 0.1
<i>MIP1/mip1^{H734Y}</i>	7.4 ± 0.9
<i>MIP1/mip1^{G807R}</i>	1.6 ± 0.1
<i>MIP1/mip1^{E900G}</i>	0.8 ± 0.1
<i>MIP1/mip1^{A692T-E900G}</i>	2.5 ± 0.1
<i>mip1^{H734Y}/mip1^{G807R}</i>	83.9 ± 2.7
<i>mip1^{G651S}/mip1^{E900G}</i>	6.9 ± 0.5

^aThe DWM-5A strain contains pFL38 and pFL39 plasmid-borne *MIP1* and *mip1* genes, respectively, as reported under Materials and Methods. Experimental conditions were as described in Table 2.

Table 4. Synergistic effects of the G807R and H734Y mutations

Haploid heteroallelic strain	% <i>petites</i>	% <i>rho</i> ⁰	Ery ^R mutant frequency (x10 ⁸)
<i>MIP1/MIP1</i>	1.0 ± 0.1	11	2.2 ± 0.4
<i>MIP1/pFL39</i>	2.7 ± 0.3	24	8.0 ± 0.8
<i>MIP1/mip1</i> ^{G807R}	1.7 ± 0.3	21	6.2 ± 0.5
<i>MIP1/mip1</i> ^{H734Y}	6.7 ± 0.7	22	3.5 ± 0.3
<i>mip1</i> ^{H734Y} / <i>mip1</i> ^{H734Y}	97.8 ± 0.2	91	ND
<i>mip1</i> ^{G807R} / <i>mip1</i> ^{G807R}	8.7 ± 1.8	29	48.6 ± 3.6
<i>mip1</i> ^{H734Y} / <i>mip1</i> ^{G807R}	70.7 ± 4.8	82	41.2 ± 4.4

The DWM-5A strain contains pFL38 and pFL39 plasmid-borne *MIP1* and *mip1* genes, respectively, as reported under Materials and Methods. Experimental conditions were as described in Table 2. ND, not determined.

Table 5. Mip1 levels and gap-filling activity in mitochondrial extracts

Strain	Mip1 level^a (% wt)	Specific gap-filling^b activity (% wt)
GALMip1	100	100
GALZ3 ^c	1.5-2.0	100-130 ^d
G651S	7-10	42-55
A692T	100	29-30
H734Y	10-20	30-46
G807R	9-15	65-86
E900G	20-50	107-139 ^d
A692T-E900G	3-6	30-40

^aThe amounts of Mip1 in the mitochondrial extracts were estimated by using Mip1 standard curves as reported in Fig.4. For each strain the extreme ranges obtained for mitochondrial extracts from different cultures are indicated.

^bWild-type GALMip1 incorporates 456 ± 50 nmol dTTP/mg extract protein/h into trichloroacetic acid precipitable material. The gap-filling activity was normalized to the same amount of Mip1 for all strains and expressed as a percentage of wild-type activity (100%).

^cGALZ3 corresponds to the wild-type strain containing the empty GALZ3 plasmid.

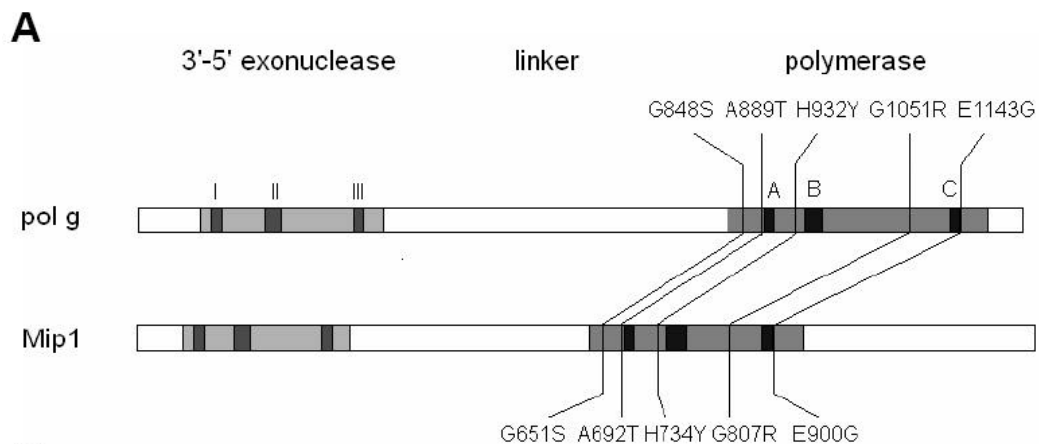
^dThese values are not significantly different from those reported for GALMip1

Table 6. *Petite* and Ery^R mutant frequency in *mip1* mutants in the T661 context

Haploid strain	% <i>petites</i>		Ery ^R mutant frequency	
	28°C	36°C	Ery ^R (x 10 ⁸)	Fold increase
<i>MIP1</i>	0.8 ± 0.1	1.0 ± 0.1	2.4 ± 0.4	1.0
<i>mip1</i> ^{G651S}	45.7 ± 4.9	99.7 ± 0.2	37.3 ± 4.7	15.5
<i>mip1</i> ^{A692T}	17.1 ± 1.6	40.1 ± 0.3	3.2 ± 0.2	1.3
<i>mip1</i> ^{H734Y}	97.8 ± 0.4	98.8 ± 0.3	ND	-
<i>mip1</i> ^{G807R}	2.1 ± 0.2	9.5 ± 0.8	7.9 ± 0.8	3.3
<i>mip1</i> ^{E900G}	1.3 ± 0.1	5.0 ± 0.1	3.8 ± 0.2	1.6
<i>mip1</i> ^{A692T-E900G}	35.5 ± 2.0	78.1 ± 3.2	3.3 ± 0.2	1.4

All experimental conditions were the same as in Table 2 except that a threonine instead of an alanine is present at position 661 of Mip1 amino acid sequence. ND, not determined.

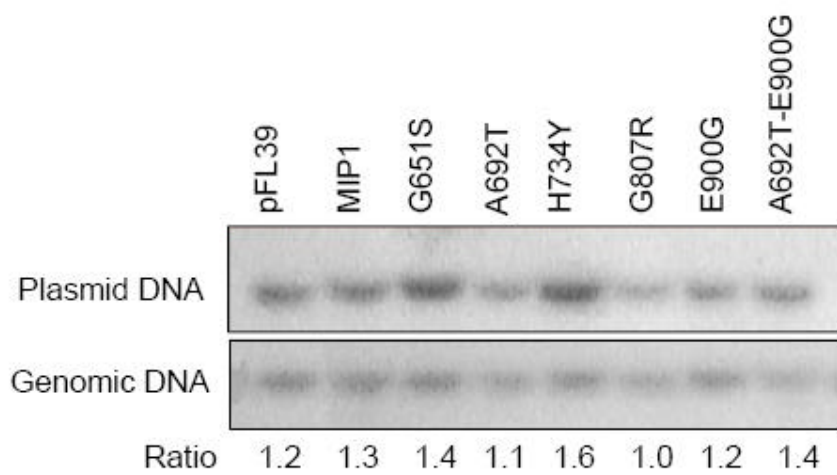
Figure 1

**B**

<i>Homo sapiens</i>	844:VVTAGTITR	885:TLVGADVDS	928:GTDLHSKTA	1047:RAWKGGTES	1139:YLVREEDRY
<i>Pan troglodytes</i>	841:VVTAGTITR	882:TLVGADVDS	925:GTDLHSKTA	1044:RAWKGGTES	1136:YLVREEDRY
<i>Mus musculus</i>	823:VVTAGTITR	864:VLVGADVDS	907:GTDLHSKTA	1026:RAWTGGTES	1118:YLVREEDRY
<i>Rattus norvegicus</i>	821:VVTAGTITR	862:VLVGADVDS	905:GTDLHSKTA	1024:RAWTGGTES	1116:YLVREEDRY
<i>Xenopus laevis</i>	814:VVSAGTITR	855:HLIGADVDS	898:GTDLHSKTA	1016:RIWTGGTES	1108:YLVHSDRY
<i>Drosophila melanogaster</i>	762:VVACGTLTR	803:RLVGADVDS	846:GSDMHSITA	951:PNWQGGTES	1043:YLVKEELSP
<i>Neurospora crassa</i>	694:VIPMGTITR	735:VPVGADVDS	777:GTDLHSRTA	853:-FWRGGTES	945:YLAEEDPKY
<i>Candida albicans</i>	721:LATMGTITR	762:CPVGADVDS	804:KTDLHSKTA	879:-IYYGGTES	971:YLVKETDKY
<i>Saccharomyces cerevisiae</i>	647:IVPMGTITR	688:CPVGADVDS	730:GTDLHKTAKTA	804:-FWYGGSES	896:PLVSEKDKY
<i>Escherichia coli</i> PolI	652:TGRVHTSYH	700:VIVSADYSQ	730:GKDIHRATA	811:-----	886:PEVHKDDV-

ACCEPTED

Figure 2



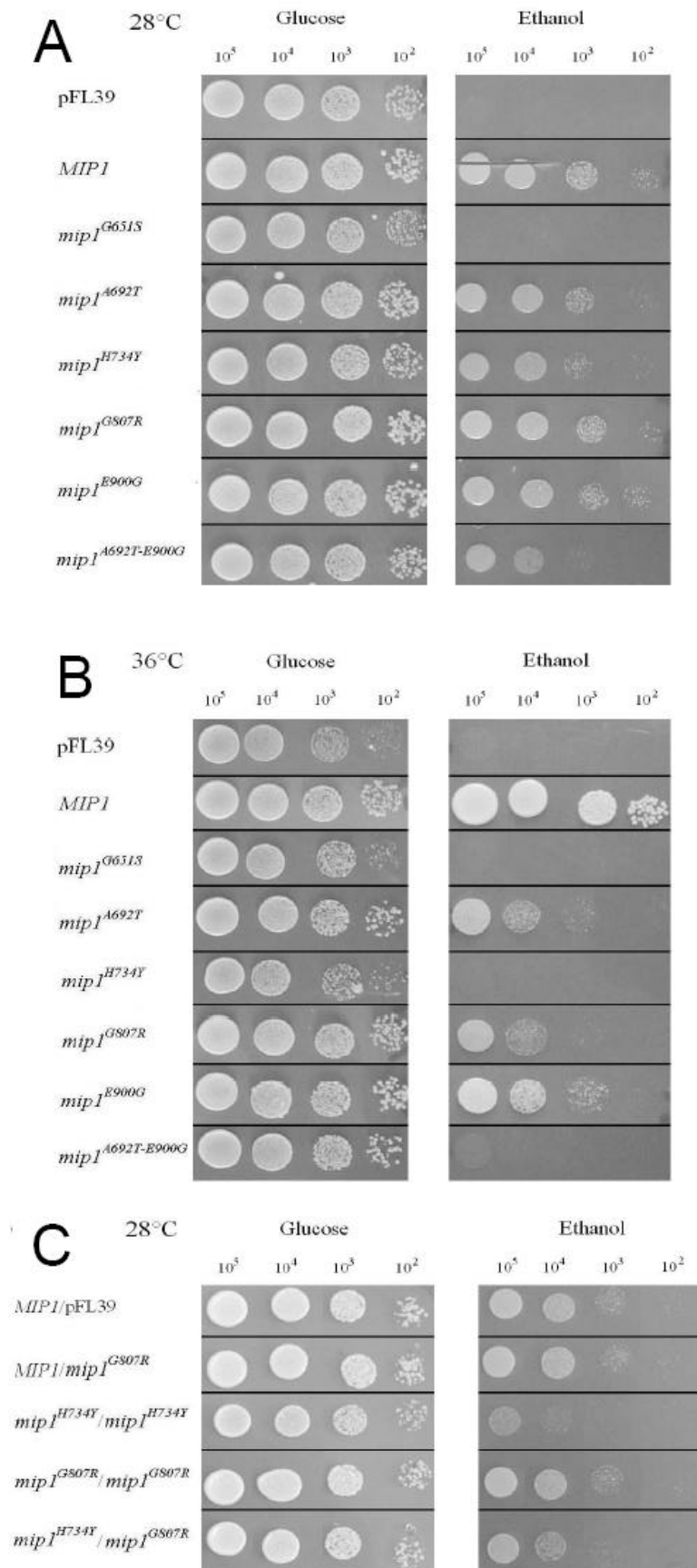


Figure 4

