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Mutation in *MTO1* involved in tRNA modification impairs mitochondrial RNA metabolism in the yeast *Saccharomyces cerevisiae*

Xinjian Wang^a, Qingfeng Yan^a, and Min-Xin Guan^{a,b,*}

^a Division of Human Genetics, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, ML 7016, Cincinnati, Ohio 45229, USA

^b Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229, USA

Abstract

The yeast *MTO1* gene encodes an evolutionarily conserved protein for the biosynthesis of the 5-carboxymethylaminomethyl group of cmnm⁵s²U in the wobble position of mitochondrial tRNA. However, *mtol* null mutant expressed the respiratory deficient phenotype only when coupled with the C1409G mutation of mitochondrial 15S rRNA. To further understand the role of *MTO1* in mitochondrial RNA metabolism, the yeast *mtol* null mutants carrying either wild-type (P^S) or 15S rRNA C1409G allele (P^R) have been characterized by examining the steady-state levels, aminoacylation capacity of mitochondrial tRNA, mitochondrial gene expression and petite formation. The steady-state levels of tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Arg} and tRNA^{Phe} were decreased significantly while those of tRNA^{Met} and tRNA^{His} were not affected in the *mtol* strains carrying the P^S allele. Strikingly, the combination of the *mtol* and C1409G mutations gave rise to the synthetic phenotype for some of the tRNAs, especially in tRNA^{Lys}, tRNA^{Met} and tRNA^{Phe}. Furthermore, the *mtol* strains exhibited a marked reduction in the aminoacylation levels of mitochondrial tRNA^{Lys}, tRNA^{Leu}, tRNA^{Arg} but almost no effect in those of tRNA^{His}. In addition, the steady-state levels of mitochondrial *COX1*, *COX2*, *COX3*, *ATP6* and *ATP9* mRNA were markedly decreased in *mtol* strains. These data strongly indicate that unmodified tRNA caused by the deletion of *MTO1* gene caused the instability of mitochondrial tRNAs and mRNAs and an impairment of aminoacylation of mitochondrial tRNAs. Consequently, the deletion of *MTO1* gene acts in synergy with the 15S rRNA C1409G mutation, leading to the loss of COX1 synthesis and subsequent respiratory deficient phenotype.

Keywords

Mitochondrial tRNA; Nucleotide modification; 15S rRNA; RNA metabolism; Yeast

1. Introduction

All tRNA species contain modified nucleosides, which are derivatives of the four normal nucleotides adenosine (A), guanosine (G), uridine (U) and cytidine (C) (Björk, 1995). In particular, nucleoside at the position 34 (wobble position of anticodon) of tRNAs is more prone to modification than those at other positions of tRNA (Hopper and Phizicky, 2003). In

*Corresponding author. Address: Division of Human Genetics, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, ML 7016, Cincinnati, Ohio 45229, USA. Tel.: +1 513 636 3337; fax: +1 513 636 2261. min-xin.guan@cchmc.org (M.-X. Guan).

Escherichia coli, the hyper-modified nucleoside 5-methyl-aminomethyl-2-thio-uridine ($\text{mnm}^5\text{s}^2\text{U34}$) occurs at the wobble position of tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} (Hopper and Phizicky, 2003). The synthesis of $\text{mnm}^5\text{s}^2\text{U}$ is a complicated process with the multiple steps in bacterial tRNAs (Brégeon et al., 2001). The products of *trmE* (Cabedo et al., 1999), *gidA* (homolog of *MTO1*) (Brégeon et al., 2001) and *trmU* (Kambampati and Lauhon, 2003; Yan et al., 2005) are components of the enzyme complex for the biosynthesis of $\text{mnm}^5\text{s}^2\text{U34}$ in the tRNAs (Björk, 1995). This modified nucleoside plays an important role in the structure and function of tRNAs, including stability, turnover, aminoacylation of tRNAs and codon recognition (Alexandrov et al., 2006; Hagervall and Björk, 1984; Sullivan et al., 1985).

Mto1p/GidA is involved in the biosynthesis for the C5 modification of $\text{mnm}^5\text{s}^2\text{U34}$ of tRNA^{Lys} , tRNA^{Glu} and tRNA^{Gln} in *E. coli* and yeast mitochondria (Yim et al., 2006; Umeda et al., 2005; Li et al., 2002, 2003). *E. coli* *gidA* mutants exhibited alterations in biosynthesis of $\text{mnm}^5\text{s}^2\text{U34}$ and translational frameshift (Brégeon et al., 2001; Yim et al., 2006). In *Saccharomyces cerevisiae*, *mto1* null mutants conferred the complete loss of 5-carboxymethylaminomethyluridine ($\text{cmnm}^5\text{s}^2\text{U}$) of mitochondrial tRNA^{Lys} (Umeda et al., 2005). Furthermore, *mto1* alleles altered the processing of mitochondrial mRNA and rRNA precursors and synthesis of COXI (Colby et al., 1998). As a result, the *S. cerevisiae* *mto1* null mutant expressed the respiratory deficient phenotype only when coexisting with a paromomycin resistance (P^{R}) C1409G mutation of mitochondrial 15S rRNA, at the very conservative decoding site, corresponding to human deafness-associated mitochondrial 12S rRNA C1494T mutation (Yan et al., 2005; Zhao et al., 2004). These strongly indicate that the product of *MTO1* functionally interacts with the decoding region of 15S rRNA, particularly at site of C1409G or A1491G mutation. To understand the role of *MTO1* in mitochondrial RNA metabolism, the yeast *mto1* null mutants have been characterized by examining the steady-state level and aminoacylation capacity of tRNA, the stability of mRNAs, and the frequency of *petite* formation.

2. Materials and methods

2.1. Yeast strains and growth analysis

Genotypes of strains were: M12 (a, *ilv5*, *trp2*, *MTO1*), M12-54 (a, *ilv5*, *trp2*, *MTO1* [P^{R}]) (Decoster et al., 1993), ΔMTO1 P^{S} (a, *his3-1*, *leu2-3*, *trp1*, *ura3-1*, *mto1::URA3*), and $\Delta\text{MTO1P}^{\text{R}}$ (a, *leu2-3*, *trp1*, *ura3-1*, *mto1::URA3* [P^{R}]) (Colby et al., 1998). The wild-type ρ^0 strains YPH500 (a, *ade2-1*, *his3-1*, *ura3-1*, *trp1*) and 15B-6B (a, *ade2-1*, *ura3-1*, *trp1*, *leu2-3*, *his3*) were isolated as described elsewhere (Wang et al., 2007). These strains carried either paromomycin sensitivity (P^{S}) or paromomycin resistance 15S rRNA C1409G allele (P^{R}). These yeast strains carried either wild-type paromomycin sensitivity mtDNA (P^{S}) or a paromomycin resistance C1409G allele (P^{R}) (Wang et al., 2007). The mitochondrial genomes of the strains were identical, apart from the C1409G allele. The complete media for yeast cell growth consists of 0.5% yeast extract Difco (Y), 1% Bacto-Peptone Difco (P), and a carbon source as specified (Guan, 1997). GYP medium contains Y, P and 2% glucose. GlyYP contains Y, P and 2% glycerol.

2.2. Petite production analysis

Yeast cells were grown in liquid GYP medium overnight, then diluted and spread on GYP medium plates. After incubation at 30 °C for 2 days, cells were replica-plated onto GlyYP medium and incubated for 4 days. Petite were scored as small colonies were grown in GYP medium but were unable to grow in GlyYP medium. At least 400 colonies for each strain from three independent cultures were scored in GYP medium. Frequency of petite formation was defined as numbers of petites/total colonies in GYP medium in each strain.

To distinguish nuclear *petite* mutants from mitochondrial *petite* mutants, 100 single clones, which were unable to grow in GlyYP medium, for each strain, were crossed to the opposite mating type wild-type ρ^+ strain YPH500 in 5% GYP medium, plated on 2% glucose minimal medium and glycerol minimal medium. After growth at 30 °C for 5 days, the diploid progeny were checked for their growth. The diploid strains showing growth on glycerol medium revealed that the nuclear gene mutation is responsible for the *petite* phenotype, while the inability to grow on glycerol medium in diploid strains indicated that these *petites* were cytoplasmic *petites* (Tzagoloff and Dieckmann, 1990).

2.3. tRNA Northern-blot analysis

Extraction of total mitochondrial RNAs, electrophoresis, hybridization and quantification of tRNA were performed as detailed elsewhere (Wang et al., 2007; Li et al., 2004). Oligodeoxynucleosides used for non-radioactive digoxigenin-(DIG) labeled probes of tRNA^{Arg2}, tRNA^{Leu}, tRNA^{Lys}, tRNA^{Gly}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Met}, and tRNA^{His}, were as detailed previously (Wang et al., 2007). Oligodeoxynucleoside for tRNA^{Phe} was 5'-TTGGTGCCCTTAATGAGAATC GAACT-3' (GenBank accession no: AJ011856) (Foury et al., 1998).

2.4. Mitochondrial tRNA aminoacylation analysis

Total mitochondrial RNAs were isolated as above but under acid condition. The total mitochondrial RNA (2 µg) was electrophoresed at 4 °C through an acid 10% polyacrylamide/7 M urea gel in a 0.1 M sodium acetate (PH 5.0) to separate the charged and uncharged tRNA, as detailed elsewhere (Enríquez and Attardi, 1996a,b). Then RNAs were electro-blotted onto a positively charged membrane (Roche) and hybridized sequentially with the specific tRNA probes as above.

2.5. Mitochondrial mRNA analysis

Total cellular RNA was obtained using a Totally RNA™ kit (Ambion) from midlog phase yeast cultures (2.0×10^7 cells) according to the manufacturer's instructions. Equal amounts (20 µg) of total RNA were fractionated by electrophoresis through a 1.8% agarose-formaldehyde gel, transferred onto a positively charged membrane (Roche Applied Science), and initially hybridized with a probe specific for *CYT8* RNA. The probe was synthesized on the corresponding restriction enzyme-linearized plasmid using a DIG RNA labeling kit (Roche Applied Science). RNA blots were then stripped and rehybridized with DIG-labeled *COX1*, *COX 2*, *COX 3*, *ATP6*, and *ATP9* probes, respectively (Yan et al., 2005; Wang et al., 2007). As an internal control, RNA blots were stripped and rehybridized with a DIG-labeled nuclear 25S rRNA probe. The plasmids used for *CYT8*, *COX1* and 25S rRNA probes were as described previously (Yan et al., 2005). The other plasmids used for mtDNA probes were constructed by PCR-amplifying fragments of *COX2* (positions 74,158–74,472), *COX3* (positions 796,084–79,967), *ATP6* (positions 28,829–29,227) and *ATP9* (positions 46,738–46,949) (GenBank accession no: AJ011856) (Foury et al., 1998), and then cloning the fragments into the pCRII-TOPO vector carrying SP6 and T7 promoters (Invitrogen).

2.6. Southern blot analysis

Yeast genomic DNA was extracted as detailed elsewhere (Sherman et al., 1986). Equal amounts (10 µg) of total DNA were digested with restriction enzyme *CfoI*, fractionated by electrophoresis through a 1.8% agarose gel, transferred onto a positively charged membrane (Roche Applied Science), and hybridized with DIG-labeled probes specific for 15S rRNA and 21S rRNA Yan et al., 2005, respectively. As an internal control, DNA blots were stripped and rehybridized with DIG-labeled nuclear 25SrRNA probe.

3. Results

3.1. Deletion of MTO1 lowered the steady-state levels of mitochondrial tRNAs

To examine if the *mtol* mutation alters the stability of mitochondrial tRNAs, the steady-state levels of the tRNAs were determined by isolating total mitochondrial RNA from four yeast strains, separating them by a 10% polyacrylamide/7 M urea gel, electroblotting and hybridizing with non-radioactive DIG-labeled oligodeoxynucleotide probes specific for tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Met}, tRNA^{Arg}, tRNA^{Phe} and tRNA^{His}, respectively. As shown in Fig. 1, the amounts of tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Arg}, tRNA^{Met} and tRNA^{Phe} were markedly decreased but those of tRNA^{His} were not affected in *mtol*^{PR} mutant cells. As shown in Table 1, the average levels of tRNAs in *mtol*^{PR} strain were 64% in the tRNA^{Lys}, 59% in tRNA^{Glu}, 41% in tRNA^{Gln}, 56% in tRNA^{Leu}, 47% in tRNA^{Gly}, 55% in tRNA^{Met}, 56% in tRNA^{Arg}, 45% in tRNA^{Phe} and 112% in tRNA^{His} of control levels (M12) after normalization to 23S rRNA, respectively. Furthermore, the average levels of tRNAs in *mtol*^{PS} strain are 79% in the tRNA^{Lys}, 59% in tRNA^{Glu}, 65% in tRNA^{Gln}, 58% in tRNA^{Leu}, 61% in tRNA^{Gly}, 99% in tRNA^{Met}, 44% in tRNA^{Arg}, 83% in tRNA^{Phe} and 136% in tRNA^{His} of control levels (M12) after normalization to 21S rRNA, respectively. It is worth noting that cells carrying *mtol* and C1409G double mutations exhibited lower steady-state levels of tRNA^{Lys}, tRNA^{Met} and tRNA^{Phe} than those in cells carrying sole *mtol* or C1409G mutation.

3.2. Deletion of MTO1 impaired aminoacylation of mitochondrial tRNAs

It has been shown that the mnm⁵s²U34 serves as a determinant for tRNA recognition by cognate aminoacyl-tRNA synthetases in *E. coli* (Hagervall and Björk, 1984; Umeda et al., 2005). This led us to test whether the *mtol* mutation affects the aminoacylation of mitochondrial tRNAs. For this purpose, the aminoacylation capacity of mitochondrial tRNA^{Lys}, tRNA^{Leu}, tRNA^{Arg} and tRNA^{His} in wild-type and mutant strains were carried out by the use of electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged tRNA species from the corresponding charged tRNA (Enríquez and Attardi, 1996a,b). As shown in Fig. 2, the upper band represented charged tRNA, and the lower band was uncharged tRNA. Electrophoretic patterns showed that either charged or uncharged tRNA^{Lys}, tRNA^{Leu} and tRNA^{Arg} in *mtol* strains migrated faster than those of *MTO1* strains, while there were no obvious differences in electrophoretic mobility of tRNA^{His} between the *mtol* and *MTO1* strains. The proportions of aminoacylation of tRNA in *MTO1*^{PS} strain were 41%, 38% and 44% in the tRNA^{Lys}, tRNA^{Leu} and tRNA^{Arg}, respectively, while the proportions of aminoacylation of the tRNAs in strains carrying *MTO1*^{PR}, *mtol*^{PS} and *mtol*^{PR} alleles: were 32%, 33% and 25% of tRNA^{Lys}, 32%, 27% and 26% of tRNA^{Leu}, 38%, 31% and 27% of tRNA^{Arg}, respectively. Aminoacylation capacity of these tRNAs were significantly decreased in *mtol* cells carrying PS or PR allele, relative to *MTO1* cells carrying PS allele ($P = 0.01-0.05$). However, there was no effect in aminoacylation efficiencies of tRNA^{His} among these control and mutant cells.

3.3. Deletion of MTO1 altered the expression of mitochondrial genome

In the previous studies, it was showed that the *mtol* mutant exhibited lower levels of mature *CYTB* and *COX1* mRNA and accumulated unprocessed or partially processed precursors in the presence of the 15S rRNA C1409G allele (Colby et al., 1998). We here tested whether the *mtol* null mutation also affected the expression of other mitochondrial genes by Northern-blot analysis. RNA blots were hybridized with DIG-labeled probes for *COX1*, *COX2*, *COX3*, *ATP6*, and *ATP9*, respectively. As an internal control, RNA blots were stripped and rehybridized with the DIG-labeled nuclear-encoded 25S rRNA probe. As shown in Fig. 3A, the amounts of mature *COX1* mRNA as well as *COX2*, *COX3*, *ATP6* and *ATP9* mRNA were decreased in the *mtol*^{PR} strain. The average levels of each mRNA were normalized to the average levels in the same strain for the 25S rRNA. As shown in Fig. 3B, the average steady-

state levels of most mRNAs were decreased in the *mtol* mutant cells, relative to the *MTOI*/ P^S strain. The steady-state levels of mRNAs in *mtol*/ P^R cells were 14% in mature *COX1*, 84% in *COX2*, 48% in *COX3*, 41% in *ATP6* and 24% in *ATP9* of control levels, while the steady-state levels of mRNAs in *mtol*/ P^S cells were 19% in mature *COX1*, 81% in *COX2*, 62% in *COX3*, 46% in *ATP6* and 37% in *ATP9* of control levels. It was noted that the levels of mature *COX1* mRNA in *mtol* cells with P^S or P^R allele were only 14% and 19% of *MTOI*/ P^S , while the levels of this mRNA in *mtol*/ P^S cells was comparable with those of controls in the previous study (Colby et al., 1998). This discrepancy is likely due to these *mtol* strains derived from different nuclear background.

3.4. Deletion of *MTOI* increased the petite production

The previous study indicated that the *mtol* mutant in the 15S rRNA P^R allele exhibited an inability to grow on glycerol medium, respectively, suggesting that the *mtol* mutation increased the petite formation (Colby et al., 1998). The rates of *petite* production in the *MTOI* strain carrying P^S or P^R allele were 3% and 20%, respectively, while the rates of petite production in the *mtol* mutant carrying P^S or P^R allele were 40% and 99%, respectively. To test if this phenotype was stable, *mtol*/ P^S respiratory negative and positive single colonies were grown in liquid glucose medium overnight, diluted, then spread on glucose medium plate. After incubation for 2 days, cells were replica-plated on glycerol medium. Among 400 single colonies from each strain, all progeny from respiratory negative strain remained the respiratory negative, while ~40% of progeny from respiratory positive strain became respiratory negative. To distinguish nuclear *petite* mutants from cytoplasmic *petite* mutants in *mtol* strains, approximately 100 single colonies showing no growth in glycerol medium were crossed to the opposite mating type wild-type p^o strains. Diploid progeny were checked for their growth in glycerol and glucose media. Diploid strains showing growth on glycerol medium revealed that the *mtol* mutation was responsible for *petite* phenotype, while the inability to grow on glycerol medium in diploid strains indicated that the *petites* were cytoplasmic *petites* (Tzagoloff and Dieckmann, 1990). Of these, the cytoplasmic *petites* account for 97%, 3% and 54% of total petite production in the *MTOI* strain carrying the P^R allele, the *mtol* strain carrying P^S or P^R allele, respectively.

3.5. Southern blot analysis

To examine if the deficient RNA metabolism caused by the inactivation of *MTOI* altered mitochondrial genomes, Southern blot analysis were determined by isolating total mitochondrial DNA from four yeast strains, digesting with restriction enzyme *CfoI*, separating them by electrophoresis, blotting and hybridizing with non-radioactive DIG-labeled 15S rRNA and 21S rRNA, respectively. As an internal control, DNA blots were stripped and rehybridized with a ^{32}P -labeled nuclear 25S rRNA probe. Quantification of the hybridization was carried out by the Image-Quant program. For comparison of the data from different blots, the values obtained for the each strain in each blot were normalized to the values obtained for the M12 sample in the same blot. Using 15S rRNA as a probe, the average levels of mtDNA in *MTOI*/ P^R , *mtol*/ P^S , *mtol*/ P^R strains were 80%, 74% and 78% of control levels (M12) after normalization to 25S rRNA, respectively. Similarly, using 21S rRNA as a probe, the average levels of mtDNA in *MTOI*/ P^R , *mtol*/ P^S , *mtol*/ P^R strains were 82%, 57% and 65% of control levels (M12) after normalization to 25S rRNA, respectively.

4. Discussion

In this study, we investigated the role of *MTOI* in mitochondrial RNA metabolism. The *E. coli* *gidA* mutants (homolog of *MTOI*) were defective in the biosynthesis of the hyper-modified nucleoside mnm⁵s²U34 (Brégeon et al., 2001). This modified nucleotide, found in the wobble position of several bacterial tRNAs specific for glutamate, lysine, and glutamine, has a pivotal

role in the function of tRNAs, including both tRNA identity and the codon recognition specificity (Björk, 1995; Li et al., 2002, 2003). In fact, the inactivation of *MTO1* in *S. cerevisiae* caused the complete loss of $\text{cmnm}^{\text{5s}^2}\text{U}$ of tRNA^{Lys} (Umeda et al., 2005). The deficiency of this nucleotide modification may alter structure and function of tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, including the stability, aminoacylation of tRNA and codon–anticodon interaction, thereby affecting the fidelity and efficiency of mitochondrial translation. These unmodified tRNAs caused by the deletion of *MTO1* may then make tRNA more unstable, reducing levels of these tRNAs. Here, the steady-state levels of tRNA^{Lys}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Arg} and tRNA^{Phe} were decreased significantly while those of tRNA^{Met} and tRNA^{His} were not affected in the *mtol* strains carrying P^S allele. In fact, lowered levels of steady-state tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln} were consistent with the loss of C5 modification in these tRNAs (Umeda et al., 2005). Nucleosides at position 34 are uridine for tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, tRNA^{Leu} and tRNA^{Gly}, cytidine for tRNA^{Met}, adenosine for tRNA^{Arg} and guanosine for tRNA^{Phe} and tRNA^{His}, respectively (Björk, 1995; Foury et al., 1998). The protein encoded by *MTO1* is involved in uridine modification at position 34 of tRNA^{Lys}, tRNA^{Glu} and tRNA^{Gln}, while other enzymes may be responsible for nucleoside modifications at this position of other tRNAs such as tRNA^{Leu}, tRNA^{Gly}, tRNA^{Met}, tRNA^{Arg}, tRNA^{Phe} and tRNA^{His} (Björk, 1995). Deletion of *MTO1* likely causes directly or indirectly transcriptional/translational defects, thereby reduces the steady-state levels of those tRNAs. Alternatively, the reduced levels of tRNA^{Leu}, tRNA^{Gly}, tRNA^{Met}, tRNA^{Arg}, tRNA^{Phe} could be attributed to preferential deletion of the ρ^- cells containing particular segments of mtDNA carrying these tRNA genes. In contrast with *mtol2* strains (Wang et al., 2007), the combination of *mtol* and C1409G mutations produced the potential synthetic phenotype for tRNA^{Lys}, tRNA^{Met} and tRNA^{Phe}. Indeed, the C1409G mutation disrupted a C1409-1491G base-pairing at the decoding site of ribosomes where the codon–anticodon recognition occurs (Yan et al., 2005). Hyper-modified tRNAs were less efficient for the decoding of codons ending in G than C of the 15S rRNA (Yan et al., 2005; Weiss-Brummer and Huttenhofer, 1989). Therefore, the C1409G allele, acting in synergy with the *mtol* mutation, may worsen deficient mitochondrial tRNA metabolisms. However, the steady-state levels of mitochondrial tRNA^{His} even increased in *mtol* cells carrying the C1409G mutation, as compared with those in P^S allele. Likely, the increased levels of tRNA^{His} could be attributed to preferential accumulation of the ρ^- cells containing particular segments of mtDNA.

The $\text{mnm}^{\text{5s}^2}\text{u34}$ is known to function as a recognition element for tRNA by cognate aminoacyl-tRNA synthetases (Krüger and Sørensen, 1998). Here, mitochondrial tRNA^{Lys}, tRNA^{Leu} and tRNA^{Arg} were uncharged to a significantly high degree *in vivo* in *mtol* strain carrying P^S allele, compared to the *MTO1*/P^S strain. Unlike the *mtol2* strains (Wang et al., 2007), the P^R allele worsened the deficient aminoacylation of these tRNAs in *mtol* cells. In addition, both charged and uncharged tRNA^{Lys}, tRNA^{Leu} and tRNA^{Arg} in *mtol* strains migrated faster than those of *MTO1* strains, while there were no obvious differences in electrophoretic mobility between wild-type and *mtol2* strains (Wang et al., 2007). Thus, *mtol* cells exhibited more inefficient aminoacylation of tRNA^{Lys}, tRNA^{Leu} and tRNA^{Arg} than those in *mtol2* cells. However, there was no difference in the aminoacylated levels of mitochondrial tRNA^{His} between the *mtol* mutant and wild-type strains.

Furthermore, the deletion of *MTO1* altered mitochondrial mRNA metabolism. Indeed, the steady-state levels of *COX1*, *COX2*, *COX3*, *ATP6* and *ATP9* mRNAs were decreased in *mtol* cells. The level of mature *COX1* mRNA in *mtol* cells was markedly decreased, but the levels of its precursors appeared to be unchanged. This strongly suggested that in the case of this transcript, the main effect was related to mRNA processing through expression of maturases encoded by mitochondrial genomes. The synthesis of maturases required for the removal of introns from *CYTb* and *COX1* (Dieckmann and Staples, 1994) was impaired by translational defects (Yan et al., 2005; Colby et al., 1998; Decoster et al., 1993). Thus, introns

in mitochondrial genomes were not spliced completely, thereby causing the accumulation of unprocessed or partially processed precursors of *CYTB* and *COX1* in *mtol* cells in the context of C1409G allele, as in the cases of *mss1* and *mtol2* cells (Yan et al., 2005; Colby et al., 1998). Of other transcripts, *COX2*, *COX3* and *ATP9* transcripts were least affected. These transcripts are parts of polycistronic transcripts containing tRNA genes, while *ATP6* lies in a primary polycistronic transcript downstream of the *COX1* gene (Tzagoloff and Dieckmann, 1990; Dieckmann and Staples, 1994). However, *COX2* transcript was not transcribed together with any tRNA gene (Dieckmann and Staples, 1994). Therefore, the lowered levels of these mitochondrial transcripts are likely due to the fact that the significant fraction of the cells, which were ρ^-/ρ^0 *petites*, did not contain templates for the transcription of relevant genes.

Mutations in genes affecting mitochondrial translation caused the instability of mtDNA, thus producing respiratory deficient phenotypes (Myers et al., 1985). Here, failures in RNA metabolism led to the heterogeneity phenotype (40% *petite* formation) in *mtol1/PS* strain, as in the case of *mtol2/PS* strain (Wang et al., 2007). This was further supported by the fact that all progeny from respiratory negative *mtol1/PS* strain remained the respiratory negative, while approximately 40% of progeny from respiratory positive *mtol1/PS* strain became respiratory negative. Indeed, the 15S rRNA C1409G mutation altered aminoacylation of tRNAs (Wang et al., 2007) and mitochondrial translation (Yan et al., 2005), consequently contributing to mtDNA instability. This was supported by the Southern blot analysis that *mtol1* mutants exhibited lower levels of mtDNA than those in *MTOL1/PS* cells. Thus, *mtol1* and C1409G double mutations led to the loss of respiration (Colby et al., 1998).

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Fig. 1. Northern-blot analysis of mitochondrial tRNA. RNA blots were hybridized with DIG-labeled oligonucleoside probes for tRNA^{Ly}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Leu}, tRNA^{Gly}, tRNA^{Met}, tRNA^{Phe}, tRNA^{Arg}, and tRNA^{His}, respectively.

Fig. 2.

In vivo aminoacylation assays for mitochondrial tRNA. (A) Two microgram of total mitochondrial RNA were treated with electrophoresis at 10% polyacrylamide/7 M urea gel, electro-blotted onto a membrane, and hybridized with DIG-labeled oligonucleoside probes for tRNA^{Lys}, tRNA^{Leu}, tRNA^{Arg} and tRNA^{His}, respectively. (B) Quantification of the levels of aminoacylated tRNA^{Lys}, tRNA^{Leu}, tRNA^{Arg} and tRNA^{His} were based on samples from three independent cultures of each tRNA in each strain.

Fig. 3.

Northern-blot analysis of mitochondrial mRNAs. (A) RNA blots were hybridized with DIG-labeled COX1, COX2, COX3, ATP6, and ATP9 probes, respectively. After re-stripping, blots were hybridized with a DIG-labeled nuclear 25S rRNA probe as internal control. (B) Quantification of the levels of mRNAs. Average relative mRNA content per cell was normalized to the average content per cell of 25S rRNA. Values were expressed as percentages of the average values for the wild strain. Samples from three independent cultures of mRNA content and 21S rRNA for each strain were used in the calculations.

Table 1

Quantification of the levels of mitochondrial tRNAs.

	<i>MTO1</i> (P ^S)	<i>MTO1</i> (P ^R)	<i>mtol1</i> (P ^S)	<i>mtol1</i> (P ^R)
tRNA ^{Lys}	100 ± 5	96 ± 8	79 ± 13	64 ± 5
tRNA ^{Glu}	100 ± 4	61 ± 6	59 ± 6	59 ± 4
tRNA ^{Gln}	100 ± 5	66 ± 7	65 ± 8	41 ± 5
tRNA ^{Leu}	100 ± 6	72 ± 6	58 ± 4	56 ± 6
tRNA ^{Gly}	100 ± 8	97 ± 7	61 ± 8	47 ± 8
tRNA ^{Arg}	100 ± 4	62 ± 6	44 ± 6	56 ± 8
tRNA ^{Met}	100 ± 12	105 ± 19	99 ± 16	55 ± 7
tRNA ^{Phe}	100 ± 4	81 ± 8	83 ± 8	45 ± 6
tRNA ^{His}	100 ± 7	81 ± 6	136 ± 5	112 ± 11

Average relative each tRNA content per cell was normalized to average content per cell of 21S rRNA. The values were expressed as percentages of the average values for the wild strain *MTO1* (P^S). Calculations were based on of each tRNA in each strain.